Formation of toxic disinfection by-products during chemomechanical preparation of infected root canals and alternative methods for root canal disinfection

Ioannidis, Kostas

Awarding institution:
King's College London

Formation of toxic disinfection by-products during chemomechanical preparation of infected root canals and alternative methods for root canal disinfection

Konstantinos Ioannidis

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ABSTRACT

Objectives: The main objective of this research was to assess the formation of toxic volatile organic compounds (VOCs) and disinfection by-products (DBPs) following the interaction of sodium hypochlorite (NaOCl) with a model system of different sources of natural organic matter (NOM) present in infected root canals, including dentine powder, planktonic multi-microbial suspensions, bovine serum albumin and their combination. A novel tooth model was further developed to study the apical extrusion of VOCs and DBPs as well as their release as effluents, during instrumentation and irrigation of artificially infected root canal specimens, with 2.5% NaOCl and 17% ethylenediaminetetraacetic acid (EDTA). Finally, this research aimed to examine alternative concepts of root canal disinfection, following the preparation of silver nanoparticles (AgNPs) synthesized on an aqueous graphene oxide (GO) matrix (Ag-GO), with different irrigant delivery methods to enhance the disinfection regimen, using a novel ex vivo infected tooth model.

Materials and methods: Dual proof of concept experiments were conducted to study the short-term oxidising effect of NaOCl at different concentrations and volumes on the structural integrity of mineralised dentine powder (MDP), at different masses, with the aid of attenuated total reflection Fourier Transform Infrared Spectrometer (ATR/FTIR). To examine the formation of chlorinated DBPs, the aliquots obtained from samples of MDP mixed with NaOCl or ultrapure water (UPW) were stirred for different time internals, from 30s to 90min. The analysis of the compounds was performed with gas chromatography coupled to a mass spectrometer equipped with electron impact ionisation source (GC-EI-MS). The use of selected ion flow tube mass spectrometry (SIFT-MS) was found appropriate to analyse the chemical derivatives present in aliquots, obtained from the real time interaction of 2.5% NaOCl with combined sources of NOM present in infected root canals. Samples were stirred at 37^0C in aerobic and anaerobic conditions for 30min to approximate a clinically realistic time. Single-rooted human teeth were decoronated to obtain 15mm-long root specimens and working length was determined 1mm short of root apex. All specimens were initially preflared to create a standardised conical space for the inoculation of the root canals with 5 selected endodontic pathogens and the development of a nutrient-stressed multispecies biofilm. Then they were randomly assigned into three groups. In group1, no endodontic intervention was performed (control). In group2, root specimens were instrumented

with rotary files and irrigated with sterile saline. In group3, root specimens were instrumented with rotary files and irrigated with 2.5% NaOCl and 17% EDTA. A customised experimental model apparatus was fabricated for each specimen, with the apical root third inserted in a glass vial filled with sterile ultrapure water, to simulate high-compliance periradicular space. A portable suction was used to aspirate the effluent during irrigation procedures. The reaction products of the aliquots obtained from the glass vials and the collected effluents were analysed in real time, by selected ion flow tube mass spectrometry (SIFT-MS) in triplicates. The aliquots obtained from group3 were further treated with silver-impregnated activated carbon (Ag-AC) to examine the effect of Ag-AC on the residual chlorine availability as well as the reduction of forming VOCs and DPBs. For the development of Ag-GO, AgNPs were prepared by reducing $AgNO_3$ with 0.01M $NaBH_4$ in presence of GO. Elemental analysis was performed with scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS) and scanning transmission electron microscopy (STEM) was used for size and morphology analysis of GO and AgNPs. Nutrient stressed, multi-species biofilms were grown in prepared root canals of single-rooted teeth. The tested irrigants were sterile saline, 1% and 2.5% sodium hypochlorite (NaOCl), 2% chlorhexidine gluconate (CHX), 17% EDTA and an aqueous suspension of 0.25% Ag-GO. The antimicrobial efficacy of root canal irrigants was performed with paper point sampling and measurement of quantitative microbial counts. The biofilm disruption capacity on dentine surfaces was analysed with confocal laser scanning microscopy (CLSM).

Results: With regard to the proof of concept studies, NaOCl concentration, MDP mass, irrigant volume and their interaction had a significant effect on the values of Amide I intensity peaks ($P<0.05$). The use of GC-EI-MS disclosed the presence of chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) within blank NaOCl solutions, as well as following the incubation with MDP, at varying concentrations, at all exposure times. SIFT-MS analysis showed the formation of several types of VOCs and DPBs, including chlorinated hydrocarbons, particularly chloroform, together with unexpected higher levels of some nitrogenous compounds, especially acetonitrile. No difference was observed between aerobic and anaerobic conditions. The chemomechanical preparation of root canals in groups 2 and 3 resulted in the apical extrusion of VOCs and DBPs. In group3, the aliquots obtained from periradicular space and the collected effluent released high concentrations of methanol, propanol, ammonia, chloroform, together with unexpected higher

levels of formaldehyde, which were statistically significant compared to group2 ($P<0.05$). In group1, VOCs and DBPs were not detected. The use of Ag-AC efficiently reduced the concentration levels of chloroform, but did not affect the presence of ammonia and formaldehyde. SEM/EDS analysis confirmed impregnation of Ag within the GO matrix. TEM images showed edge-shaped GO sheets and spherical AgNPs of diameter 20-50nm, forming a network on the surface of GO sheets. The microbial killing efficacy of 2.5% NaOCl was superior compared to the experimental groups. The use of ultrasonic activation enhanced the efficacy of Ag-GO compared to 1% NaOCl, 2% CHX, 17% EDTA and sterile saline ($P<0.05$). The maximum biofilm disruption, in dentine tubules, was achieved by 2.5% NaOCl. Ag-GO caused a significant reduction of total biovolumes compared to the rest experimental groups ($P<0.05\%$).

Conclusions: The chemical interaction of NaOCl with NOM resulted in the formation of toxic chlorinated hydrocarbons. SIFT-MS analysis proved to be an effective analytical method. The mechanical preparation and irrigation of artificially infected root canals with rotary NiTi files, 2.5% NaOCl and 17% EDTA resulted in the formation of VOCs and DBPs in a water-closed periradicular space and aspirated effluent aliquots. The risks from the rise of toxic compounds require further consideration in dentistry. The biofilm killing and disruption capacity of Ag-GO was successfully documented in a novel ex vivo infected tooth model. Ultrasonic activation selectively improved the antimicrobial efficacy of Ag-GO.

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List of Abbreviations

Abbreviation	Meaning
A	Amide I
AC	Activated Carbon
AgNPs	Silver nanoparticles
Ag-AC	Silver impregnated activated carbon
Ag-GO	Ag-NPs synthesized on an aqueous GO matrix
ANOVA	Analysis of variance
ANP	Apical negative pressure
AOM	Algal organic matter
ATR-FTIR	Attenuated total reflectance Fourier transform-infrared spectroscopy
ATSDR	Agency for Toxic Substances and Disease Registry
BCME	Bis(chloromethyl)ether
BHI	Brain heart infusion
BSA	Bovine serum albumin
C	Carbonate
Ca	Calcium
CFD	Computational fluid dynamics
CFU	Colony forming units
CH_3CHO	Methanol
$CHCl_3$	Chloroform
CH_3CN	Acetonitrile
CHX	Chlorhexidine
CI	Conventional irrigation

CLSM	Confocal laser scanning microscopy
CT	Computed tomography
CUI	Continuous ultrasonic irrigation
CWAIS	Continuous warm activated irrigation and evacuation system
DBP	Disinfection by-product
DOC	Dissolved organic carbon
DW	Distilled water
D50	Mean diameter
ECD	Electron-capture detector
ECHA	European Chemicals Agency
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EfOM	Effluent organic matter
EPS	Extracellular polymeric substances
ESE	European Society of Endodontology
EU	European Union
FAA	Fastidious anaerobe agar
FS	Full scan
FTIR	Fourier transform-infrared
GC-ECD	Gas chromatography-electron caption detector
GC-EI-MS	Electron impact gass chromatography mass spectrometry
GC-MS	Gas chromatography–mass spectrometry
GO	Graphene oxide
HA	Hydroxyapatite

HAA	Haloacetic acid
HMDS	Hexamethyldisilazane
HEBP	Etidronic acid
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
H_3O^+	Hydronium
IARC	International Agency for Research on Cancer
IPCS	International Programme on Chemical Safety
LAI	Laser activated irrigation
LAT1	Apical third lateral canal
LAT2	Middle third lateral canal
MDW	mass ratio-distilled water
MFUM	Modified fluid universal medium
MIM	Multiple-ion monitoring
MW	Molecular weight
M/Z	Mass to charge ratio
$NaBH_4$	Sodium borohydrate
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NCLP	Non-contacting laser profilometry
NH_3	Ammonia
NIST	National Institute of Standards and Technology
NiTi	Nickel-titanium

NCPs	Non-collagenous proteins
NOM	Natural organic matter
NO^+	Nitrosonium
NMR	Nuclear magnetic resonance
N/D	Non-detectable
NP	Nanoparticle
OCl^-	Anion hypochlorite
OSHA	Occupational Safety and Health Administration
O_2^+	Dioxygenyl ion
P	Phosphate
PBS	Phosphate buffered saline
PCA	Para-chloroaniline
Pd-AC	Activated carbon loaded with palladium
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PGs	Proteoglycanes
PIPS	Photon-induced photoacoustic streaming
PLM	Polarised light microscopy
POU	Point-of-use
PUI	Passive ultrasonic irrigation
P/A	Phosphate to Amide ratio
P/C	Phosphate to Carbonate ratio
RR	Reaction rate
RT	Retention time

SAF	Self-adjusting file
SD	Standard deviation
SEM	Scanning electron microscopy
SEM/EDS	Scanning electron microscopy/energy dispersive spectroscopy
SH-	Sulphydryl groups
SIFT-MS	Selected ion flow tube mass spectrometry
SIM	Single ion monitoring
SMPs	Soluble microbial products
SPME	Solid phase microextraction
TEM	Transmission electron microscopy
THM	Trihalomethane
TLC	Thin layer chromatography
TOF-SIMS	Time-of-flight secondary ion mass spectrometry
TUWA	Ultrasonic wave aspiration
UAI	Ultrasonically activated irrigation
USEPA	United States Environmental Protection Agency
VDW	Volume of distilled water
VOC	Volatile compound
WHO	World Health Organization
WL	Working length
XPEF	XP Endo finisher
XPS	X-ray photon spectroscopy

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Introduction

Overview

Bacterial growth within a necrotic root canal is the main cause of apical periodontitis (Kakehashi *et al.* 1965, Möller *et al.* 1981). The goal of root canal treatment is to prevent or facilitate the healing of apical periodontitis and maintain the function of root canal treated teeth (Ørstavik & Pitt Ford 2008). A successful endodontic treatment outcome is a result of a combination of several clinical procedures including mechanical preparation, irrigation, disinfection, three-dimensional obturation of the root canal system and a coronal restoration (Torabinejad *et al.* 2005). Periradicular tissue healing is dependent on adequate elimination or reduction of bacterial population (Dalton *et al.* 1998) and the presence of residual biofilms in un-instrumented root canal walls is a common cause of root canal treatment failure and post-treatment apical periodontitis (Ricucci *et al.* 2013).

NaOCl (1-5%) is currently regarded as the gold standard for irrigation and chemical disinfection of the root canal system (Sedgley 2004). The use of a demineralizing agent such as 17% EDTA is further recommended for the removal of the contaminated inorganic content of the smear layer, which is deposited on dentine walls during instrumentation (Patterson 1963). Irrigant agitation and activation techniques may be necessary to aid the dispersal of the irrigant into the root canal system (Macedo *et al.* 2014) and minimise the negative effect of irrigant stagnation and apical vapor lock within the confinements of a close root canal system (Mohhmed *et al.* 2016).

Despite its beneficial antimicrobial and solubilising capacity, NaOCl is known for its deleterious effects against dentine collagen (Oyarzún *et al.* 2002) and its caustic effects on soft and hard tissues (Pashley *et al.* 1985, Kerbl *et al.* 2012). In addition, when NaOCl reacts with compounds of the infected root canal, toxic volatile compounds (VOCs) and chlorinated disinfection by-products (DBPs) are also formed (Varise *et al.* 2014), however, there is currently very limited dental literature. NaOCl is one of the most widely used components in water disinfection to prevent the spread of microbial or viral diseases and contamination and maintain public health (Khan *et al.* 2016). Despite its importance, chlorination of water for disinfection may result in the formation of potentially harmful DBPs, due to the reaction of chlorine or hypochlorite

with natural organic matter (NOM) (Krasner 2009, Chen & Westerhoff 2010). Frequent exposure to chlorinated water via inhalation, drinking, dermal absorption through bathing, showering and swimming (Lin & Hoang 1999, Backer *et al.* 2000, Ashley *et al.* 2005, Chowdhury *et al.* 2014), can lead to DBP formation and may be dangerous to human health because of the carcinogenic and mutagenic properties of these compounds (Villanueva *et al.* 2006, Richardson *et al.* 2007).

To address biocompatibility issues, biofilm resistance and anatomical restrictions, it is essential to search for new endodontic biomaterials to promote root canal disinfection. The incorporation of nanoparticles (NPs) has gained attention in the past few decades due to their innovative and functional properties (Abeylath & Turos 2008). Nanoparticles of silver (Ag NPs) are well-known for their antibacterial capacity (Choi & Hu 2008, Rai *et al.* 2009). Carbon materials are known to be more environmentally and biologically friendly than inorganic materials, since the carbon is one of the most common elements in our ecosystem. Graphene oxide (GO) is a promising material for biological applications (Chung *et al.* 2013) and exhibit antibacterial activity against several bacterial species (Nanda *et al.* 2016). Recent studies have shown that a hybrid biomaterial of Ag NPs, where a layered material like GO is used as matrix, can induce binding capability, that is usually lacking with Ag NPs alone and thus is able to enhance antimicrobial action (Das *et al.* 2011, Song *et al.* 2016).

Research aims

1. The aim of the first proof of concept study was to compare *ex vivo* the dentine mass- and irrigant volume- dependent effects of NaOCl on the structural integrity of dentine. The aim of the second proof of concept study was to examine the formation of chlorinated DBPs when NaOCl interacts with dentine *ex vivo* in short and long exposure times.
2. The aim of the first main study was to assess the formation of VOCs and DBPs from the interaction between 2.5% v/v NaOCl and variable components of an artificially infected root canal system.
3. The aim of the second main study was to assess the release of VOCs and DBPs during instrumentation and irrigation of artificially infected root canal specimens, with 2.5% NaOCl and 17% EDTA, in a novel tooth model.

4. The aim of the third main study was to apply powdered Ag-AC in post-chlorinated aliquots obtained from the previous study and assess the dechlorinating capacity as well as the removal capacity against VOCs and DBPs.
5. The purpose of fourth study was to examine the antimicrobial efficacy of silver nanoparticles (AgNPs) synthesized on an aqueous graphene oxide (GO) matrix (Ag-GO), with different irrigant delivery methods to enhance the disinfection regimen, using a novel *ex vivo* infected tooth model.

Structure of the thesis

The thesis is divided into seven chapters and follows a systematic investigation of the interaction of sodium hypochlorite with dentine using state-of-the-art analytical techniques, followed by alternatives for disinfection of the root canal and the efficacy studied using a newly proposed model.

Chapter 1 is a review of the literature and discusses various aspects of root canal irrigation including: The main types of root canal irrigants and their mechanism of action, the principles of irrigant flow and delivery methods, the role of irrigant agitation, the interaction of NaOCl and other irrigants with various components of an infected root canal system, such as bacteria, biofilms, pulpal tissue and dentine. Additionally, the risk of inadvertent apical extrusion is discussed, as well as the chemical interactions between root canal irrigants. Finally, the scientific knowledge from literature regarding the formation of toxic chlorinated DBPs and the limited dental literature on this topic are critically appraised, among with the need to implement modern approaches for the disinfection of root canals with the application of NPs.

Chapter 2 investigates the effects of NaOCl on organic and inorganic components of mineralised dentine using ATR-FTIR, as well as describes a preliminary report on the formation of chlorinated DBPs, following chemical analysis with GS-EI-MS.

Chapter 3 examines the methodological suitability of SIFT-MS for the real-time analysis of volatile compounds released after the chemical interaction of 2.5% NaOCl with representative components of an infected root canal system.

Chapter 4 describes the fabrication of a novel tooth model and its application to assess the apical extrusion and effluent release of VOCs and DBPs during instrumentation and irrigation of artificially infected root canal specimens, with 2.5% NaOCl and 17% EDTA.

Chapter 5 discusses the application of Ag-AC for the elimination of VOCs and DBPs in effluents produced, following aspiration of NaOCl with dental suctioning.

Chapter 6 examines the antimicrobial efficacy of silver nanoparticles (AgNPs) synthesized on an aqueous graphene oxide (GO) matrix (Ag-GO), with different irrigant delivery methods to enhance the disinfection regimen, using a novel ex vivo infected tooth model.

Chapter 7 describes the main findings of the four studies, along with future work that could be carried out on this research topic.

Chapter 1 Literature Review

1.1 Root canal treatment

According to the European Society of Endodontology (ESE), 'Endodontology is concerned with the study of the form, function and health of, injuries to and diseases of the dental pulp and periradicular region, their prevention and treatment' (ESE 2006). Endodontic treatment encompasses procedures that are designed to maintain the health of all or part of the dental pulp. In addition, the aim of root canal treatment is to preserve or restore periradicular tissues back to health and preserve functional teeth without prejudice to the health of the patient (ESE 2006).

A successful endodontic treatment outcome is a result of a combination of several clinical procedures including mechanical preparation, irrigation, disinfection and three-dimensional obturation of the root canal system (Torabinejad *et al.* 2005). After this, a permanent coronal restoration can be placed to restore function and aesthetics (Figure 1-1). The creation of a coronal and apical hermetic seal through adequate root filling and coronal restoration should result in prevention of future reinfection (Ray & Trope 1995, Hülsmann *et al.* 2005).

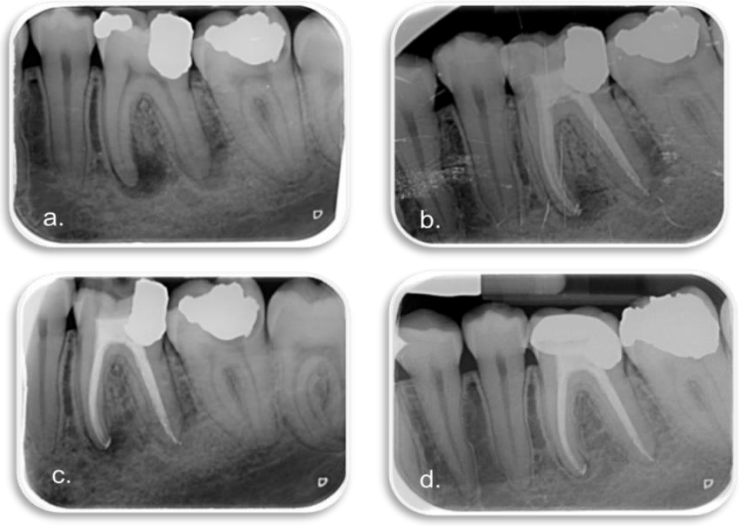


Figure 1-1 Phases of a root canal treatment. (a) A LL6 diagnosed with pulp necrosis and associated chronic apical periodontitis; (b) Single-visit chemomechanical preparation, root canal obturation and permanent composite core placement; (c) 6-month review-established periapical healing is noticeable; (d) 12-month review- Cuspal coverage and protection with a crown and complete resolution of apical radiolucency and bone regeneration is noticed.

1.1.1 Mechanical preparation

The mechanical preparation of the root canals allows for the removal of the majority of the root canal contents in the main root canal space (Schilder 1974), including inflamed pulp (vital and necrotic tissues), bacteria and infected dentine. In addition, the forming root canal shape allows the facilitation of intracanal medicaments and the obturation of the root canal system in three dimensions (Peters 2004). The modern concept of root canal treatment additionally considers mechanical preparation as a measure to facilitate delivery and exchange of the antimicrobial agents to the crucial apical root third (Gulabivala *et al.* 2010).

However, the complex anatomy of root canal system, tends to leave up to 50% of canal walls un-instrumented during preparation, potentially resulting in insufficient debridement (Peters 2001, Peters 2004, Peters *et al.* 2010). An example of cases with anatomical complexities is presented in Figure 1-2. Necrotic tissue remnants may act as a source of nutrition for any surviving bacteria in inaccessible areas and the presence of residual pulp tissue, infected dentine, or bacteria in the root canal system are contributing factors leading to treatment failure (Makinen & Makinen 1996, Love 2001). Therefore, root canal irrigation plays an indispensable role in root anal disinfection of several parts, complexities and canal irregularities (Figure 1-3), that are inaccessible for mechanical preparation.

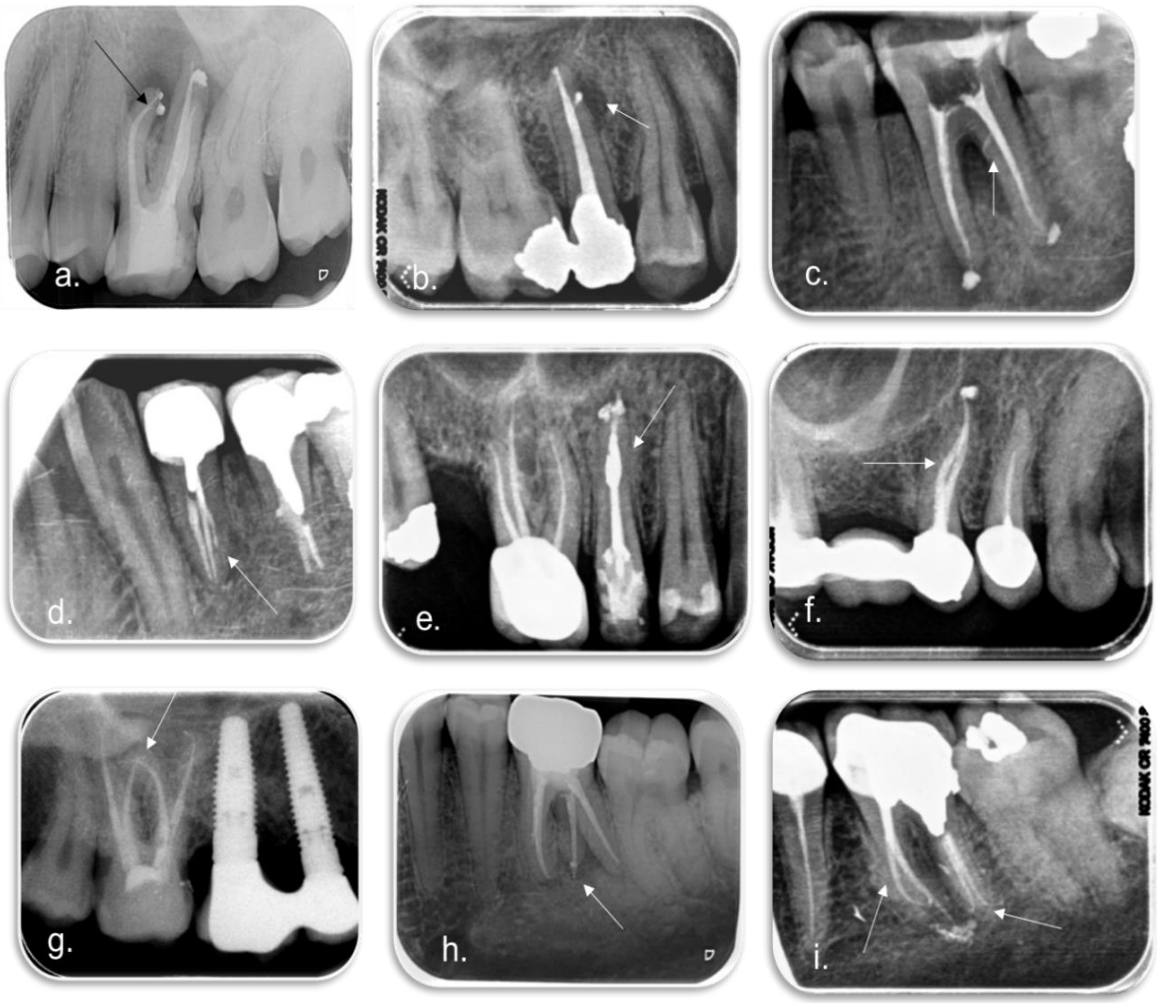


Figure 1-2 Periapical radiographs which present anatomical challenges of the root canal system (from KI personal archive). (a) Apical delta in an UL6; (b) Apical lateral canal in an UR5; (c) Coronal lateral canal and furcation involvement in a LL6; (d) Three separate canals in a LL4; (e) Internal root resorption in an UR5; (f) S-shaped root canals in an UR5; (g) Two independent palatal roots in an UR7; (h) radix entomolaris in a LL6; (i) Mesial and distal roots with a main root canal orifices dividing in two independent canals in middle root third.

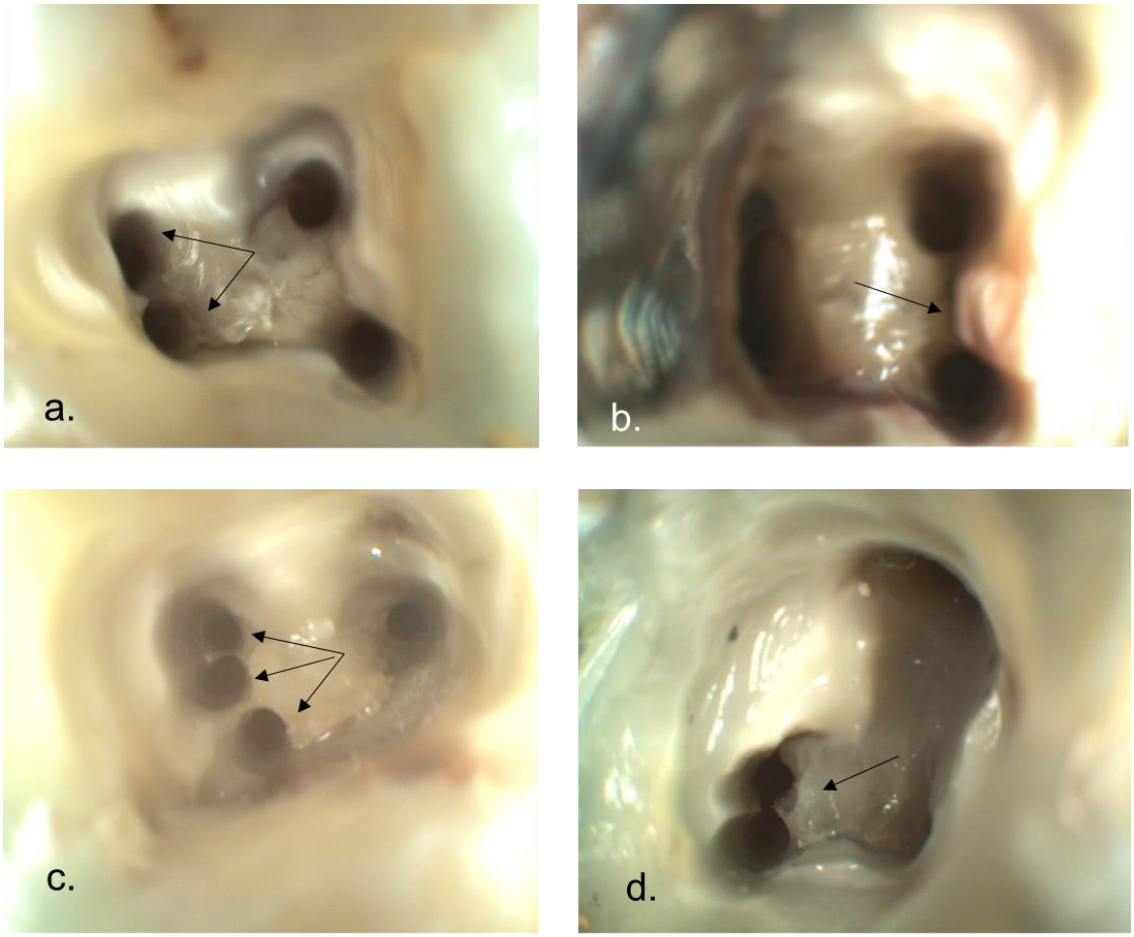


Figure 1-3 Clinical images of disinfected pulp chambers obtained from lower and upper molars with the aid of dental microscope (from KI personal archive). (a) Two independent root canal orifices in distal root of lower molar; (b) Presence of isthmus in the mesial root canals of a lower molar; (c) Three root canal orifices and presence of middle mesial canal in a lower molar; (d) MB1 and MB2 root canals in an upper molar.

1.1.2 Root canal irrigation

Root canal irrigation can be defined as the procedure to deliver a liquid or irrigant in the root canal system before, during and after instrumentation of the root canal. Instrumentation of the root canal system must always be supported by irrigation to fulfil additional mechanical, chemical and biological objectives.

The efficacy of an irrigant depends on its capacity to flush out debris, lubricate the canal, dissolve organic and inorganic tissue and prevent the formation of a smear layer during instrumentation or remove it once it has formed. Moreover, root canal irrigants must have a wide-spectrum antimicrobial effect against bacteria in their planktonic state and in biofilms, inactivate endotoxins and remain non-toxic in contact with periodontal and periapical tissues (Zehnder 2006, Basrani & Haapasalo 2013).

1.2 Sodium hypochlorite (NaOCl) in endodontic procedures

1.2.1 History of chlorine-releasing agents

Potassium hypochlorite was the first chemically produced aqueous chlorine solution, invented in France by Berthollet (1748-1822). Starting in the late 18th century, hypochlorite solutions were used as bleaching agents and industrially produced by Percy in Javel near Paris, hence the name “Eau de Javel”. NaOCl was recommended by Labarraque (1777-1850) to prevent childbed fever and other infectious diseases. Koch and Pasteur further investigated NaOCl as a potent disinfectant by the end of the 19th century.

In World War I, the chemist Henry Drysdale Dakin and the surgeon Alexis Carrel extended the use of a buffered 0.5% NaOCl solution to the irrigation of infected wounds, based on Dakin’s meticulous studies on the efficacy of different solutions on infected necrotic tissue (Dakin 1915). Hypochlorite preparations were also found to be sporicidal, virucidal and showed greater tissue dissolving effects on necrotic than on vital tissues (McDonnell & Russell 1999). These features prompted the use of aqueous NaOCl in endodontics as the main irrigant as early as 1919 as recommended by Coolidge (Coolidge 1929).

1.2.2 Mechanism of action of NaOCl

NaOCl is a strong non-specific oxidizing agent. When dissolved in water it slowly decomposes, releasing chlorine, oxygen, sodium and hydroxyl ions (Figure 1-4) (Estrela *et al.* 2002).

$$4\ NaOCl + 2\ H_2O \rightarrow 4\ Na^+ + 4\ OH^- + 2\ Cl_2 + O_2$$

or

(1) $NaOCl + H_2O \leftrightarrow NaOH + HOCl$

(2) $NaOH\ (aq) \leftrightarrow Na^+ + OH^-$

(3) $HOCl\ (aq) \leftrightarrow H^+ + OCl^-$

Figure 1-4 Decomposition of NaOCl in aqueous solution (adopted by Estrela *et al.* 2002).

In aqueous solution, hypochlorous acid (HOCl) (equation 3) partially dissociates into the anion hypochlorite (OCl^-). The "available" chlorine is the sum of the HOCl and OCl^- concentrations in a solution (Baker 1947). HOCl has stronger chlorinating and oxidizing capacity on tissue and microorganisms than OCl^-. The dissociation of HOCl (equation 3) depends on pH and the maintenance of the clinical equilibrium between HOCl and OCl^- is achieved once HOCl is consumed through its antimicrobial function (Baker 1947, Baker 1959, Winter *et al* .2008).

During root canal chemomechanical preparation, NaOCl comes in contact with the contents of the root canal system, which comprise of microbial biofilms/endotoxins/metabolic products, vital or necrotized cellular structures, blood and plasma, tissue exudates, vital healthy, inflamed or necrotic pulp tissue, root dentine, vital or non-vital odontoblasts and overall organic and inorganic matter and debris formed after mechanical instrumentation (Torabinejad *et al.* 2002).

Sodium hypochlorite, in contact with organic materials causes different chemical reactions that depend on the available reactive species in the aqueous environment. Sodium hydroxide (NaOH) forms reacts with fatty acids and amino acids leading to the formation of soap and glycerol as well as salt and water, respectively (Figure 1-5; Schemes 1 & 2) (Estrela *et al.* 1995).

HOCl reacts with amino acids leading to chloramine formation. Further reaction of chloramines with HOCl and OCl^- leads to hydrolysis and degradation of amino acids, formation of volatile dichloramines and nitrogen trichloride (Figure 1-5; Scheme 3). Chloramines are able to interfere with cellular metabolism leading to irreversible oxidation of SH- (sulphydryl groups) of essential bacterial enzymes (Baker 1959). When NaOCl contacts tissue proteins, within a short time nitrogen, formaldehyde, and acetaldehyde are formed. The peptide links are broken up to dissolve the proteins (Basrani & Haapasalo 2012).

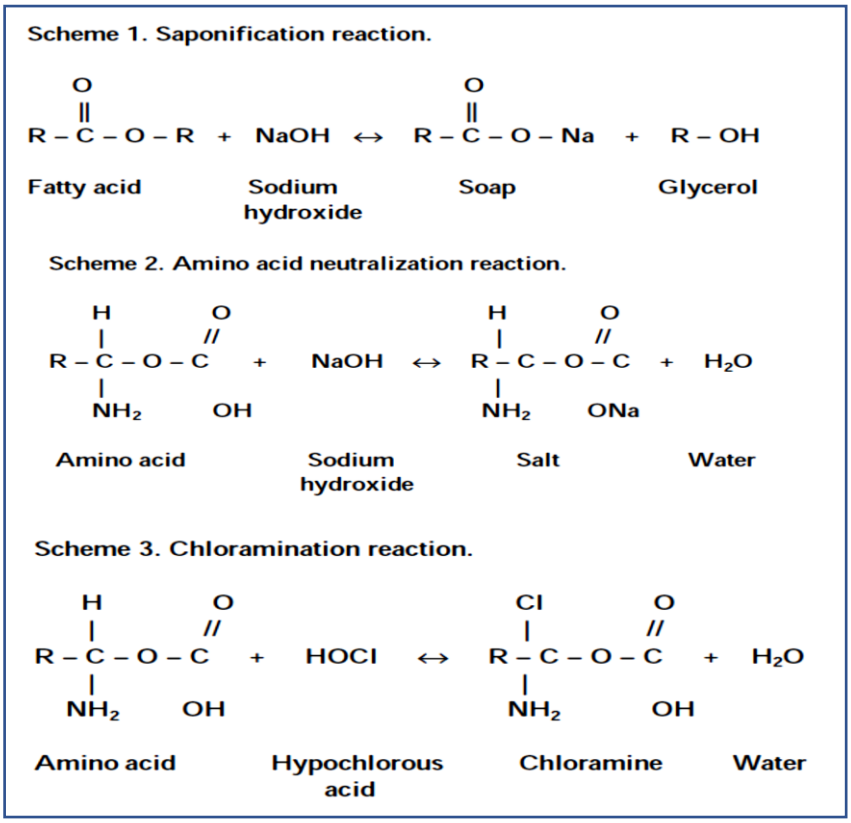


Figure 1-5 Chemical reactions of NaOCl with organic material including fatty acids and aminoacids (adopted by Estrela *et al.* 2002).

1.2.3 Concentration of sodium hypochlorite in endodontic use

Since 1920, NaOCl has been universally adopted and extensively used in endodontics as an irrigant for disinfection of root canals, where concentrations range between 1% and 5.25%. This is due to its antimicrobial properties (Byström & Sundqvist 1983, Byström & Sundqvist 1985, Soares & Junior 2006, Davis *et al.* 2007, Siqueira *et al.* 2010), its ability to disintegrate and solubilize organic tissue (Senia *et al*. 1971, Koskinen *et al*. 1980, Naenni *et al.* 2004, Clarkson *et al.* 2006, Combakara *et al* .2010) and to denature toxins (Buttler & Crawford 1982). In addition, it is inexpensive, has a long shelf life and is readily available (Johnson & Reimekis 1993, Frais *et al.* 2001).

There is no general consensus regarding the concentration of NaOCl solutions e used in endodontics. As Dakin's original 0.5% NaOCl solution was designed to treat open (burnt) wounds, it was surmised that in the confined area of a root canal system, higher concentrations should be used, as they would be more efficient than Dakin's solution (Grossman 1943). The existing controversy has always been discussed as a matter of balancing the antibacterial effectiveness and tissue dissolution capacity, whilst considering its negative effects on dentine's biomechanical properties, cellular cytotoxicity and caustic effects (Spångberg *et al.* 1973, Sedgley 2004).

In modern dentistry, the use of NaOCl is often limited to 1% to minimise potential damage when reaching the periapical tissue. Clinical studies have indicated that the bactericidal effects of 1% NaOCl have shown not to be significantly lower than concentrations as high as 5.25%, when using sufficient volumes (Cvek *et al.* 1976, Bystrom & Sundqvist 1981, Siqueira *et al.* 2000). It must be realized that during irrigation, fresh NaOCl should constantly reach the canal system and concentration of the solution may not play a decisive role (Siqueira *et al.* 2007). Unclean areas may be a result of the inability of solutions to physically reach these areas rather than their concentration (Senia *et al.* 1971, Moorer & Wesselink 1982). However, it has also been shown, both through *in vitro* studies and case reports, to be toxic to the periapical tissues, especially when applied at high concentrations (Önçağ *et al.* 2003, Gernhardt *et al.* 2004). Furthermore, a 5.25% solution significantly decreases the elastic modulus and flexural strength of human dentine compared to physiologic saline, while a 0.5% solution does not, after 30min of interaction (Table 1-1) (Sim *et al.* 2001). This is most likely because of the proteolytic action of concentrated hypochlorite on the collagen matrix of dentine and will be further discussed in the followingchapters. Hence, based on the currently available evidence, there is no rationale for using NaOCl solutions at concentrations over 1% v/v.

Table 1-1 Mean (SD) of modulus of elasticity and flexural strength for each group (adopted by Sim *et al.* 2001).

Groups	**n**	**Modulus of elasticity (Nm^{-2}) Mean (SD)**	**Flexural strength (Nm^{-2}) Mean (SD)**
Saline	37	15.1E + 9 (2.1E + 9)	17.0E + 7 (4.5E + 7)
NaOCl 0.5%	31	14.5E + 9 (2.8E + 9)	14.8E + 7 (4.0E + 7)
NaOCl 5.25%	32	12.9E + 9 (2.3E + 9)	10.3E + 7 (3.0E + 7)

1.3 Chlorhexidine (CHX) in endodontic procedures

1.3.1 History

Chlorhexidine was developed in the late 1940s in the research laboratories of Imperial Chemical Industries (Macclesfield, England). Since 1957 it has been used for general disinfection purposes and also for the treatment of skin, eye, and throat infections in both humans and animals (Löe 1973, Fardal & Turnbull 1986).

1.3.2 Mechanism of action of CHX

CHX belongs to the poly-biguanide antibacterial family, consisting of two symmetric 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain (Greenstein *et al.* 1986). It is a strong base and is most stable in the form of its salts. The original salts were CHX acetate and hydrochloride, both of which are relatively poorly soluble in water (Foulkes 1973). Hence, they have been replaced by CHX digluconate.

CHX is a wide-spectrum antimicrobial agent, active against Gram-positive and Gram-negative bacteria, and yeasts (Mohammadi & Abbott 2009). Due to its cationic nature, CHX is capable of electrostatically binding to the negatively charged surfaces of bacteria, damaging the outer layers of the cell wall and rendering it permeable (Davies 1973). Depending on its concentration, CHX can have both bacteriostatic and bactericidal effects. At high concentrations, CHX acts as a detergent and by damaging the cell membrane, it causes precipitation of the cytoplasm and thereby exerts a bactericidal effect (Hugo & Longworth 1966). At low sub-lethal

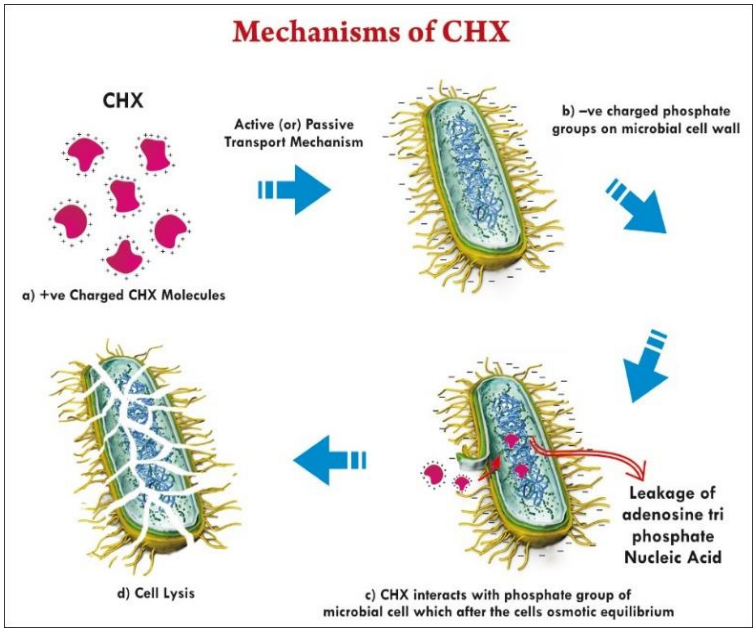


Figure 1-6 Mechanism of antimicrobial action of CHX (adopted by http://kvabpharm.com/xlorgeksidin-mexanizm-dejstvija.html, accessed on 25-3-2019).

concentrations, chlorhexidine is bacteriostatic, causing low molecular weight substances, i.e. potassium and phosphorous, to leak out without the cell being irreversibly damaged. It also can affect bacterial metabolism in several other ways such as abolishing the activity of the PTS sugar transport system and inhibiting acid production in some bacteria (Gomes *et al.* 2001, Gomes *et al.* 2003).

1.3.3 Concentration for endodontic use

CHX is a potent antiseptic, which is widely used for chemical plaque control in the oral cavity (Addy & Moran 1997). Aqueous solutions of 0.1 to 0.2% are recommended for this purpose, while 2% is the concentration of root canal irrigating solutions usually found in the endodontic literature (Zamani *et al.* 2003).

CHX as well as tetracyclines have a unique characteristic when they react with dentine. Medicated dentine with CHX acquires antimicrobial substantivity (Khademi *et al.* 2006). The positively charged ions released by CHX can adsorb on dentine and prevent microbial colonization on the surface for some time beyond the actual time of application of the medicament (Athanassiadis *et al.* 2007). Therefore, CHX was originally proposed as a final irrigant, due to its antimicrobial substantivity.

In a laboratory study, White *et al.* (1997) evaluated the antimicrobial substantivity of a 2% CHX solution as an endodontic irrigant. Findings showed that the substantivity lasted 72h. However, other studies have reported that the substantivity of CHX can last for longer periods of time. Khademi *et al.* (2006) found that 5min application of 2% CHX solution induced substantivity for up to 4 weeks whilst Rosenthal *et al.* (2004) evaluated the substantivity of 2% CHX solution within the root canal system after 10min of application and they reported that the CHX was retained in the root canal dentine in antimicrobially effective amounts for up to 12 weeks. In an *in vivo* study, Leonardo *et al.* (1999) evaluated the antimicrobial substantivity of 2% CHX used as a root canal irrigating solution in teeth with pulp necrosis and radiographically visible chronic periapical lesions. They reported that the CHX prevented microbial activity with residual effects in the root canal system for up to 48h after application.

On the contrary, Lin *et al.* (2003) attributed the substantivity of CHX to its ability to adsorb on dentine during the first hour. They stated that it is only after the saturation point is reached after the first hour that the antimicrobial capability of CHX increases with time. Furthermore, Komorowski *et al.* (2000) showed that 5min application of CHX did not induce substantivity and that the dentine should be treated with CHX for 7 days.

Many randomized clinical studies evaluated the antimicrobial effectiveness of CHX 2% compared to 1% or 2.5% NaOCl, during preparation and irrigation of human infected root canals, in both primary and retreatment cases. Using DNA extraction and PCR methods, they found an equal reduction of bacterial counts, regardless of irrigant selection (Rôças *et al.* 2016, Zandi *et al.* 2016). On the other hand, many clinicians support that CHX cannot be advocated as the main irrigant in standard endodontic cases, due to lack of dissolution capacity against vital and necrotic tissue remnants (Naenni *et al.* 2004) and the lack of antimicrobial effectiveness against Gram-negative, compared to Gram-positive bacteria (Emilson 1977, do Amorim *et al.* 2004, Hashemi *et al.* 2019). In a randomized clinical trial on the reduction of intracanal microbiota by either 2.5% NaOCl or 0.2% CHX irrigation, it was found that NaOCl was significantly more efficient than CHX in obtaining negative cultures (Ringel *et al.* 1982). This was especially the case for anaerobic bacteria, while the difference for facultative taxa was less significant. Furthermore, more culture reversals from negative to positive were found with CHX than with NaOCl. The authors attributed this phenomenon to the inability of CHX to dissolve necrotic tissue remnants and chemically clean the canal system.

1.4 The role of chelation in endodontic procedures

1.4.1 Formation of smear layer during root canal instrumentation

The identification of the smear layer was made possible using the electron microprobe with scanning electron microscope (SEM) attachment, and was first reported by Boyd *et al.* (1963). Later, Eick *et al.* (1970) showed initially that the smear layer was made of particles of 0.5–15μm in size. Scanning electron microscope images of cavity preparations by Brännström & Johnson

(1974) demonstrated a thin layer of grinding debris. They estimated it to be 2–5μm thick, extending a few micrometres into the dentinal tubules.

The first researchers to describe the smear layer on the surface of instrumented root canals were McComb & Smith (1975). The smear layer was described as a thin, amorphous layer, 0.5–2mm thick, that covers the dentine surface. Lester & Boyde (1977) described the smear layer as 'organic matter trapped within translocated inorganic dentine'. As it was not removed by NaOCl, they concluded that it was primarily composed of inorganic dentine. Additional work has shown that the smear layer consisted not only of dentine as in the coronal smear layer, but also the remnants of odontoblastic processes, predentine, remnants of pulp tissue, bacteria and biofilm (Pashley *et al.* 1988, Torabinejad *et al.* 2002).

The generation of a smear layer is almost inevitable during root canal instrumentation (Pashley 1992). A smear layer always forms when a metallic endodontic instrument touches a mineralized dentine wall within a root canal (Mader *et al.* 1984). The smear layer is caused by hand and rotary stainless steel and nickel-titanium (NiTi) files as well as by ultrasonic tips and various burs that are used in the root canal. The consistency of the smear layer may affect a dentist's decision regarding its removal. Particularly, when we refer to cases of infected root canals, the smear layer should be removed for the following reasons:

(i) it may contain microbial cells and antigens (Haapasalo *et al.* 2012).

(ii) it weakens the effects of disinfecting agents in dentine (Ørstavik & Haapasalo 1990, Wang *et al.* 2013).

(iii) it may affect the quality of the root filling and bonding to the canal wall. Intracanal bonding depends on the type of the sealer. Most studies have shown that removal of the smear layer improves bonding of many sealers to dentine (Economides *et al.* 1999, Saleh *et al.* 2002, Eldeniz *et al.* 2005).

Because the smear layer contains both inorganic and organic material, it cannot be removed by any of the presently available root canal irrigants alone, including NaOCl. The sequential use of NaOCl and a chelating agent or acid that dissolves inorganic tissue is required.

1.4.2 Ethylenediaminetetraacetic acid (EDTA)

The compound was first described in 1935 by Ferdinand Munz, who prepared the compound from ethylenediamine and chloroacetic acid. Today, EDTA is mainly synthesized from ethylenediamine, formaldehyde and sodium cyanide (Basrani & Haapasalo 2012). It is colourless and water-soluble. The ability of chelators to bind and inactivate metallic ions is widely exploited in medicine. Chelators can be used to bring about excretion of dangerous ions in the case of metal poisoning or in the treatment of copper metabolism disturbances (Ding *et al.* 2011, Kosnett 2013). In 1951, the first reports on the demineralizing effect of EDTA on dental hard tissues were published (Hahn & Reygadas 1951, Screebny & Nikiforuk 1951). Chelators were first introduced to endodontics by Nygaard-Østby (1957), who recommended the use of a 15% EDTA solution, with pH=7.3.

1.4.3 Mechanism of action

EDTA is a polyamino-carboxylic acid with the formula $[CH_2N\ (CH_2CO_2H)_2]_2$. Its prominence as a chelating agent arises from its ability to “sequester” di- and tri-cationic metal ions such as Ca^{2+} and Fe^{3+} (Figure 1-7). After being bound by EDTA, metal ions remain in solution but exhibit diminished reactivity (Hülsmann *et al.* 2003). The term ‘chelate’ originates from the Greek word ‘chele’ (crab claw). Chelates are particularly stable complexes of metal ions with organic substances as a result of ring-shaped bonds. This stability is a result of the bond between the chelator, which has more than one pair of free electrons, and the central metal ion (Hülsmann *et al.* 2003).

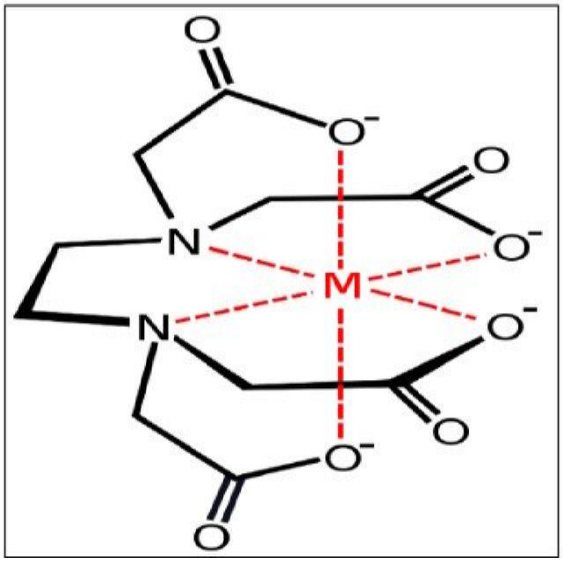


Figure 1-7 Chelation Mechanism of EDTA with metal (M).

Chelators such as EDTA form a stable complex with calcium. When all available ions have been bound, an equilibrium is formed and no further dissolution takes place. Using gravimetrical analyses, Seidberg & Schilder (1974) showed that the properties of EDTA were self-limiting. This limitation is thought to be because of pH changes during demineralization of dentine. Under neutral conditions, most chelators have a pH near the neutral value and 99% of the EDTA is present as $EDTAHNa_3$. The exchange of calcium from the dentine by hydrogen results in a subsequent decrease in pH. Because of the release of acid, the efficiency of EDTA decreases with time; on the other hand, the reaction of the acid with hydroxyapatite affects the solubility of dentine.

EDTA is universally recommended as an auxiliary irrigation solution because it can chelate and remove the mineralized portion of smear layers (Tartari *et al.* 2017). EDTA alone normally cannot remove the smear layer effectively; a proteolytic component (NaOCl) must also be used (preferably before EDTA) to remove the organic components of the smear layer (Wang *et al.* 2013). EDTA has limited antibacterial action against endodontic biofilms (Zhang *et al.* 2015). With direct exposure for an extended period of time, EDTA may extract bacterial surface proteins by combining with metal ions from the cell envelope, which can eventually lead to biofilm disruption and bacterial death (de Almeida *et al.* 2016, Liu *et al.* 2017).

1.4.4 Concentration of EDTA in endodontics

The recommended protocol for smear layer removal is irrigation with NaOCl followed by EDTA. EDTA is normally used at a concentration of 15-17%. However, the results of an *in vitro* study showed that different concentrations of EDTA (15%, 10%, 5%, and 1%) presented no difference on the smear layer removal from instrumented root canal walls (Sen *et al.* 2009). The authors concluded that lower concentrations of EDTA can be recommended for clinical usage to avoid excessive erosion of root canal dentine.

Despite the good predictability of smear layer removal, there is no consensus in terms of the exact time required for NaOCl and EDTA irrigation to completely remove it. Varying results have been reported with irrigation times usually ranging between 1 and 5min (Calt & Serper 2002,

Teixeira et al. 2005). An *in vitro* study showed that 10ml 17% EDTA was capable to remove smear layer within 1min. A 10-min application of EDTA caused excessive peritubular and intertubular dentinal erosion. Therefore, the authors suggested that this procedure should not be prolonged for more than 1min during endodontic treatment (Calt & Serper 2002).

1.5 Delivery of root canal irrigants

The effectiveness of root canal irrigation relies the ability of the irrigants to kill bacteria (Gulabivala *et al.* 2005) and dissolve the necrotic tissues (Lee *et al.* 2004). Apart from the chemical aspect of root canal disinfection, the effectiveness of irrigation relies on the mechanical flushing action (Baker *et al.* 1975). The dynamics of root canal irrigation play an important role, when setting the operating parameters to evaluate the effects of an irrigant against the root canal contents (Gulabivala *et al.* 2005). Fluid motion in a confined and complex geometry is a central problem, which makes the dispersion and mixing of irrigant more difficult because of the absence of turbulence over much of the canal volume (Gulabivala *et al.* 2010). The objectives of irrigant delivery are to induce a flow of irrigant to the full extent of the root canal system, in order to come in close contact, detach/disrupt and carry away the infected substrate, and provide lubrication for the instruments (Chávez de Paz *et al.* 2015). Irrigant flow may ensure an adequate delivery throughout the root canal system, refreshment and mixing of the irrigant, in order to compensate for its rapid inactivation (Chávez de Paz et al. 2015). In the meantime, irrigant flow should be restricted within the constraints of the root canal, to prevent extrusion towards the periapical tissues (Chávez de Paz et al. 2015).

1.5.1 Conventional manual irrigation

Classical endodontic handbooks refer to conventional manual irrigation as a ‘simple procedure’ (Ingle *et al.* 2002). Despite the provision of practical guidelines (Ruddle 2002, Wesselink & Bergenholtz 2004), the recommendations reflect a certain degree of empiricism regarding the irrigation procedure. Boutsioukis *et al.* (2007) highlighted the need for the

standardization of the procedure and the adoption of clear guidelines to improve educational and research purposes.

Conventional irrigation with syringes and needles still remains widely accepted (Ingle *et al.* 2002, Peters 2004), and has also been advocated as an efficient method of irrigant delivery prior to the development of agitation techniques such as passive ultrasonic activation (PUI) (van der Sluis *et al.* 2006). Syringe delivery of the irrigant allows control of the volume and the depth of needle penetration in the canal (van der Sluis *et al.* 2006). Different syringes of varying volume capacity from 1 to 5ml have been used in previous studies (Senia *et al.* 1971, Sedgley *et al.* 2005, Tinaz *et al.* 2005). In principle, larger capacity syringes require greater force to move the plunger and result in less control of the procedure, as a small movement of the plunger results in larger volume delivered (Boutsioukis *et al.* 2007). On the other hand, they also require less frequent refilling.

Manufacturers of the irrigation systems have furnished needles and syringes with luer-lock connectors to prevent needle detachment during use, in contrast to simple friction fitting available in normal medical syringes (Moser & Heuer 1982). The lack of a luer-lock combined with the application of high pressure could lead to barrel failure or to sudden detachment of the needle hub from the syringe (Clarkson & Moule 1998, Lambrianidis 2001). Therefore, syringes and needles with such luer-lock connectors should be preferred (Clarkson & Moule 1998, Lambrianidis 2001).

Boutsioukis *et al.* (2007) monitored and compared *ex vivo* irrigant flow rate, intra-barrel pressure, duration of irrigation and volume of irrigant delivered with a 5-ml syringe and three endodontic needles (25, 27 and 30 gauge) with luer-lock. The testing system was connected with pressure and displacement transducers into a prepared root canal and ten specialist endodontists performed and repeated the irrigation procedure ten times with each needle.

The results of the study showed that finer diameter needles required increased effort to deliver the irrigant and resulted in higher intra-barrel pressure. In addition, the use of a needle with a very fine lumen should be considered clinically impractical, because of crystallization of the NaOCl solution in the lumen during the course of treatment (Senia *et al.* 1971, Moser & Heuer 1982), which would further increase the required effort. The syringe and needles with luer-lock

tolerated the pressure developed. No detachment of needle hub or barrel failure occurred and peak intra-barrel pressures in the range of 400–550 kPa were well-tolerated by the syringe-needle systems used. Syringe irrigation was difficult to standardize and control, since wide variations of flow rate were observed among operators (Boutsioukis *et al.* 2007).

1.5.2 Irrigant flow in root canals

Irrigant flow rate should be considered as the main factor that directly influences flow beyond the needle, however it is rarely mentioned in relation to irrigation effectiveness (Williams *et al.* 1995). The optimal irrigant flow rate has not been determined yet and future studies should test the effect in clinical conditions. The cause of irrigant flow rate is associated with the pressure difference between the syringe barrel and the prepared root canal space. However, it should be noted that it does not directly affect the flow pattern within the root canal.

Many studies have highlighted the importance of needle placement close to the working length, which is the end-point of root canal preparation, in order to achieve better contact with remaining bacteria (Seal *et al.* 2002), remove intra-canal debris (Abou-Rass & Piccinino 1982, Moser & Heuer 1982, Chow 1983) and reduce bacterial counts during root canal irrigation (Sedgley *et al.* 2005). The exact knowledge of the external diameter of the needle tip is crucial for selection of needles of appropriate size. Such information is mostly unavailable and confusing, as the units of the widely used ‘gauge’ system are not directly comparable to the size of the instruments used for intracanal procedures (Zinelis *et al.* 2002).

Universally accepted medical stainless steel needle tubing dimensions are defined by the ISO 9626:1991/ Amd 1:2001 specification (ISO 9626 2001). The ISO standards refer to medical needles only, though, endodontic irrigation needles should also comply with the universal specifications. The study of Boutsioukis *et al.* (2007b) was the first to measure the internal and external diameter of several stainless steel irrigation needles with variable size 21-30G and a Ni-Ti needle (30G) under scanning electron microscopy (SEM) and stereoscopic microscopy to determine the incidence and degree of deviation from ISO 9626:1991/Amd 1:2001. None of the needles tested complied with the ISO nominal size, but stainless steel needles were within the

ISO tolerance limits. The Ni-Ti needle was found to exceed ISO external diameter limits. The tolerance of the external diameter of needles and files is importance in order to minimize the risk of binding in the root canal (Zinelis *et al.* 2002). The use of the 'gauge' system to categorize the size of the needles has been widely accepted by manufacturers as it possesses significant advantages concerning the manufacturing process (Pöll 1999). However, the units of measure cannot be directly extrapolated to clinical practice and even if the clinician is aware of the needle size appropriate for a root canal, there remains a difficulty in distinguishing the different sizes under clinical conditions (Boutsioukis *et al.* 2007b). The authors have further indicated that adoption of the millimetre (mm) size categorization as the standard metric unit should be accelerated. In addition, a colour-coding of needles corresponding to endodontic instruments was further recommended in order to establish a specific standard for endodontic needles. Since 2007, no further advancements have been documented in this domain.

The penetration of irrigant and the flushing action created by irrigation depend not only on the anatomy of the root canal system, but also on the system of delivery, the depth of placement, and the volume and fluid properties of the irrigant (Kahn *et al.* 1995, Lee *et al.* 2004, Gulabivala *et al.* 2005). The challenging environment of a complex root canal system may result in limited efficiency of root canal irrigants to reach the most apical region, resulting in poorer performance of the irrigant (Druttman & Stock 1989, Haapasalo *et al.* 2005).

Earlier macroscopic attempts to evaluate the flow of irrigants within the canal were based on the observation of the displacement of a coloured dye by the delivered irrigant (Kahn *et al.* 1995), or on radiographic appearance of a partly radiopaque solution (Ram 1977, Salzgeber & Brilliant 1977, Abou-Rass & Piccinino 1982). However, it was impossible to detect the dynamics of fluid movement in the root canal and the flow pattern around the needle.

Hsieh *et al.* (2007) showed dynamic flow conditions during irrigation, in human extracted teeth with variable apical sizes, with thermal image analysis. The flow distribution of root canal irrigation was affected adversely by large diameter irrigating needles, by greater distances between the needle tip and the apical stop and the final size of the root canal preparation. The authors suggested that the applied wind tunnel concept was a valuable model in the study of the occurring hydrodynamic changes in irrigated root canals. However, a major limitation was the

effect of the simultaneous heat flux from the warm fresh irrigant to the cooler irrigant within the canal and the canal walls.

The application of Computational Fluid Dynamics (CFD) to study the dynamics of root canal irrigation was introduced by Boutsioukis *et al.* (2009). It is regarded as a powerful tool to investigate flow patterns, velocity field, shear stress, pressure and physical and chemical phenomena by mathematical modelling and computer simulation (Tilton 1999, Arvand *et al.* 2005). The irrigant flow in a simulated canal of known dimensions (length of 19mm, apical diameter of 0.45mm) with 6% taper and closed impermeable apex, using different needle types, was investigated by an unsteady CFD model (Boutsoukis *et al.* 2010). Six different types of 30-G needles, three open-ended needles and three close-ended needles, were positioned 3mm from the closed apex and the irrigant flow in the apical root canal was calculated and visualized. The open-ended needles created a jet toward the apex and maximum irrigant replacement but also higher apical pressure. Within the close-ended group, the side-vented and double side-vented needle created a series of vortices and a less efficient irrigant replacement. The multi-vented needle created almost no flow apically to its tip, and wall shear stress was concentrated on a limited area, but the apical pressure was significantly lower than the other types.

In the same validated CFD model, Boutsioukis *et al.* (2010b) further studied the effect of apical preparation size on irrigant flows from either a side-vented or a flat 30G needle positioned inside root canals having sizes of 25, 35, 45 and 55, all with a 6% taper, at 3mm short of working length (Boutsioukis *et al.* 2010b). The side-vented needle could not achieve irrigant replacement to the WL even in a size 55, 6% taper root canal. Significant irrigant replacement was evident with the flat needle, almost to the WL in size 35, 45 and 55, 6% taper root canals. The maximum shear stress decreased as the preparation size increased. The flat needle developed higher mean pressure at the apical foramen. Root canal enlargement to sizes larger than 25 appeared to improve the performance of syringe irrigation. The authors further concluded that adequate space between the needle and the canal wall should be ensured to allow for an effective reverse flow of the irrigant towards the canal orifice (Boutsioukis et al. 2010b).

The effect of root canal taper on the irrigant flow was also investigated in a validated CFD model, which was used to simulate irrigant flow from either a side-vented or a flat 30G needle

positioned inside size 30, 2% taper, 30, 4%, 30, 6%, ProTaper F3 (variable taper) or size 60, 2% taper root canals, at 3mm short of working length (Boutsioukis *et al.* 2010c). The side-vented needle could not achieve irrigant replacement to the WL in any of the cases. Significant irrigant replacement was evident further than 2 mm apically to the tip of the flat needle in the size 30, 6%, F3 and size 60, 2%. A wider distribution of wall shear stress was noted as the canal taper increased but the maximum shear stress decreased. The flat needle led to higher mean pressure at the apical foramen. An increase in root canal taper improved irrigant replacement and wall shear stress whilst reducing the risk for irrigant extrusion. Irrigant flow in a minimally tapered root canal with a large apical preparation size also improved irrigant replacement and wall shear stress and reduced the risk for irrigant extrusion, compared to the tapered root canals with a smaller apical preparation size (Boutsioukis *et al.* 2010c).

The effect of needle-insertion depth on the irrigant flow was evaluated inside a prepared root canal during final irrigation with a syringe and two different needle types using a CFD model (Boutsioukis *et al.* 2010d). Irrigant flow, velocity, pressure and shear stress from either a side-vented or an open-ended flat 30-G needle positioned inside a prepared root canal (apical preparation size:45, 6% taper) at 1, 2, 3, 4, or 5 mm short of the working length were evaluated. Major differences were observed between the two needle types. The side-vented needle achieved irrigant replacement to the WL only at the 1-mm position, whereas the open-ended flat needle was able to achieve complete replacement even when positioned at 2 mm short of the WL. The maximum shear stress decreased as needles moved away from the WL. The flat needle led to higher mean pressure at the apical foramen.

The higher the velocity of the irrigant, the faster and more adequate the replacement is. Shear stress on the canal wall will have an influence on the mechanical detachment of debris, tissue remnants, isolated microbes, and biofilm (Boutsioukis *et al.* 2010d). Although there are no quantitative data on the minimum shear stress required, the distribution of shear stress along the canal wall provides an indication of the debridement efficacy of each needle type. It must be emphasized that the disruption or detachment of biofilm or debris cannot ensure their removal unless there is a favorable irrigant flow to carry them toward the canal orifice (reverse flow).

According to the results of the present study, positioning of the needle closer to the WL improved the irrigant replacement in the apical part of the root canal, but also led to increased mean pressure at the apical foramen, indicating an increased risk of irrigant extrusion toward the periapical tissue (Boutsioukis *et al.* 2010d). The requirements of adequate irrigant replacement and reduced apical pressure appeared to contradict each other. From a clinical point of view, the prevention of extrusion should precede the requirement for adequate irrigant replacement and wall shear stress. However, because there is no definite evidence on the minimum irrigant pressure that leads to extrusion, the risk of extrusion can only be estimated by comparison between different needle positions.

1.5.3 The role of irrigant agitation in root canal therapy

The mechanical flushing action created by conventional hand-held syringe needle irrigation is relatively weak. After conventional syringe needle irrigation, inaccessible canal extensions and irregularities are likely to harbour debris and bacteria, thereby making thorough canal debridement difficult (Wu *&* Wesselink 2001, Wu *et al.* 2006). Irrigation enhancement beyond needle irrigation is relevant to more effective eradication of microorganisms from root canal systems.

Factors that improve the efficacy of syringe needle irrigation include closer proximity of the irrigation needle to the apex (Chow 1983, Sedgley *et al.* 2005), larger irrigation volume (Sedgley *et al.* 2004), and smaller-gauge irrigation needles (Chow 1983). However, for the achievement of these conditions, root canals may require enlargement with the negative consequences in radicular dentine thickness and subsequent weakening of the root structure (Lertchirakarn *et al.* 2003), and risk of inadvertent irrigant extrusion is present if needle tips is placed short of working length. Irrigant flow rate and exchange are diffucult to standardize and control during syringe needle irrigation (Boutsioukis *et al.* 2007). Thus, it would be advantageous to develop new application systems that enhance the depth of irrigant penetration within dentinal tubules. This ensures more thorough debridement of the prepared canals, while minimizing apical extrusion to eliminate the cytotoxic effects of canal irrigants such as NaOCl on the periapical tissues, as well as safe distribution of the irrigant volumes towards the axial root canal walls (Bradford *et al.* 2002).

Throughout the history of endodontics, endeavors have continuously been made to develop more effective irrigant delivery and agitation systems for root canal irrigation. Irrigants must be brought into direct contact with the entire canal wall surfaces for effective action particularly for the apical portions of small root canals.

1.5.4 Classification of irrigant agitation and delivery systems

Irrigant agitation and delivery systems can be divided in 2 broad categories:

1. Manual agitation techniques, including manual and manual dynamic activation
2. Machine- assisted agitation techniques, including sonic and ultrasonic activation.

1.5.4.1 Manual and manual dynamic agitation techniques

Manual agitation can be achieved by dispensing of an irrigant into a canal through needles/cannulas of variable gauges, either passively or with agitation. The latter is achieved by moving the needle up and down the canal space. It is crucial that the needle/cannula should remain loose inside the canal during irrigation. This allows the irrigant to reflux and causes more debris to be displaced coronally, while avoiding the inadvertent expression of the irrigant into periapical tissues. One of the advantages of syringe irrigation is that it allows comparatively easy control of the depth of needle penetration within the canal and the volume of irrigant that is flushed through the canal (van der Sluis *et al.* 2006).

Manual dynamic irrigation has been considered as a simplistic and cost-effective, but laborious hand-activation procedure, in which a well-fitting gutta-percha master cone is gently moved up and down in short 2-3mm strokes within an instrumented canal to produce an effective hydrodynamic effect and improve the displacement and exchange of any given irrigant (McGill *et al.* 2008, Huang *et al.* 2008).

Studies have shown that manual dynamic irrigation generate higher intracanal pressure changes during pushing movements compared to static irrigation, leading to more effective delivery of irrigant to the ''untouched'' canal surfaces, creates sufficient turbulence the push-pull

motion of the gutta-percha point probably acts by physically displacing, folding, and cutting of fluid under ‘‘viscously-dominated flow’’ in the root canal system (Wiggins & Ottino 2004). The latter probably allows better mixing of the fresh unreacted solution with the spent, reacted irrigant (McGill *et al.* 2008, Huang *et al.* 2008).

However, Hülsmann *et al.* (2009) stated that, in any endodontic treatment, an instrument used in an apical direction or an instrument acting as a plunger may result in periapical extrusion of root canal biomass. This has been well documented by a recent study, in which manual dynamic irrigation extruded significantly more NaOCl in a closed periradicular space compared to ultrasonic or sonic activation (Psimma *et al.* 2014).

1.5.4.2 Sonic agitation

Tronstad *et al.* (1985) were the first to report the use of a sonic instrument for endodontics. Sonic agitation irrigation operates at a lower frequency (1–6 kHz) and produces smaller shear stresses; therefore, it is different from ultrasonic irrigation (Ahmad *et al.* 1987). The sonic energy generates a high amplitude and a great back-and-forth tip movement. The oscillating patterns of the sonic devices are different compared with ultrasonically driven instruments.

A minimum oscillation of the amplitude might be considered a node, whereas a maximum oscillation of the amplitude represents an antinode. They have 1 node near the attachment of the file and 1 antinode at the tip of the file (Walmsley *et al.* 1989). However, when the movement of the sonic file is constrained, the sideway oscillation disappears. This results in a pure longitudinal file oscillation. This mode of vibration has been shown to be particularly efficient for root canal debridement, because it is largely unaffected by loading and exhibits large displacement amplitudes (Walmsley *et al.* 1989). However, sonic devices do not display any cavitation effects around their sonically oscillating tips, even when driven at their highest frequency (Macedo *et al.* 2014).

Conventionally, sonic irrigation was performed by using a Rispisonic file attached to a MM 1500 sonic handpiece (Medidenta International, Inc, Woodside, NY) after canal shaping. The Rispisonic files have a non-uniform taper that increases with file size (Figure 1-8). However, the

file surface is barbed and these files might inadvertently engage the canal wall and damage the finished canal preparation during agitation.

Figure 1-8 The MM sonic handpiece and the rispisonic files (adopted by www.medidenta.com).

The EndoActivator System (Dentsply Sirona) is a more recently introduced sonically driven canal irrigation system with 2000–10000 cpm, meaning 33–167 Hz (Jensen *et al.* 1999, Ruddle 2008). It consists of a portable handpiece and 3 types of disposable polymer tips of different sizes (Figure 1-9). Because they are smooth, they do not cut dentine.

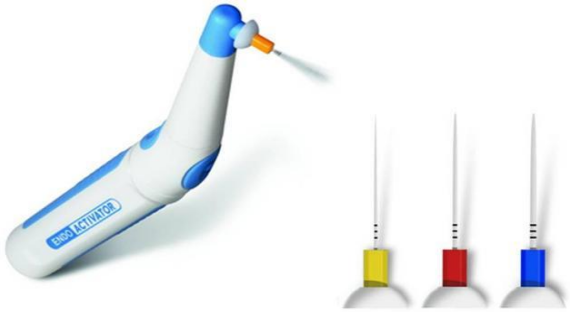


Figure 1-9. The EndoActivator system and the disposable tips of different sizes (adopted by www.denstsplysirona.com).

EDDY (VDW, Munich, Germany) is a non-cutting, sterile single-use instrument sonic powered irrigation activation system made of flexible polyamide with a size of 25.04 (Figure 1-10). According to the manufacturer, it allows an efficient cleaning of complex root canal systems without the limitations of ultrasound-activated devices. EDDY is activated with 5000 to 6000 Hz by an airdriven handpiece (Air Scaler) (VDW). The instrument is claimed to create a three-dimensional movement that triggers cavitation and acoustic streaming, two physical effects which up to now have only been caused by PUI and which have been attributed with the improved cleaning efficiency of PUI (van der Sluis *et al.* 2010). Despite the promising outcomes in canal cleanliness (Urban *et al.* 2017), further investigation is required to justify the exact pattern of cavitation and acoustic streaming.

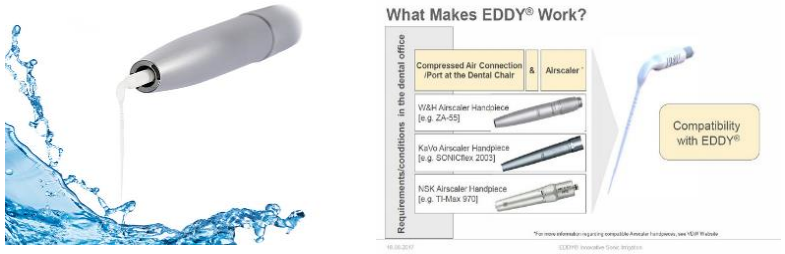


Figure 1-10 The Eddy sonic powered irrigation system (adopted by www.vdw-dental.com).

Flexible sonic tips (EDDY and EndoActivator) may have an advantage compared to commonly used rigid metal tips when performing PUI, as they may easily reach the crucial apical canal portion even in severely curved root canals and may oscillate despite contact to the canal walls. There is a consensus in literature on the superiority of sonic activation, compared to conventional irrigation, in dentine debris and smear layer removal capacity from mesial canal isthmuses (Stamos *et al.* 1987, Jensen *et al.* 1999, Urban *et al.* 2017), however when compared to ultrasonic agitation the results are conflicting (Mancini *et al.* 2013).

1.5.4.3 Ultrasonic agitation

Ultrasonic devices have long been used in periodontics and were first used in endodontics by Richman (1957), as a means of canal debridement. In 1980, Martin *et al.* designed an ultrasonic unit, which became commercially available for endodontic use. Compared with sonic energy, ultrasonic energy produces high frequencies but low amplitudes (Walmsley & Williams 1989). The files are designed to oscillate at ultrasonic frequencies of 25–30 kHz, which are beyond the limit of human auditory perception (>20 kHz). They operate in a transverse vibration, setting up a characteristic pattern of nodes and antinodes along their length (van der Sluis *et al.* 2007). During ultrasonic activation, the energy is transmitted from an oscillating file or a smooth wire to the irrigant in the root canal by means of ultrasonic waves. The latter induces acoustic streaming and cavitation of the irrigant (Ahmad *et al.* 1987, Ahmad *et al.* 1987b, Ahmad *et al.* 1988).

1.5.4.4 Acoustic microstreaming

Acoustic streaming is the rapid movement of fluid in a circular or vortex-like motion around a vibrating file (Walmsley 1987). The acoustic streaming that occurs in the root canal during

ultrasonic irrigation has been described as acoustic microstreaming. Several studies have confirmed that acoustic micro streaming occurs (Ahmad *et al.* 1987, Ahmad *et al.* 1992, Walmsley 1987, Roy *et al.* 1994). The streaming pattern corresponds to the characteristic pattern of nodes and antinodes along the length of the oscillating file. The resultant acoustic microstreaming depends inversely on the surface area of the file touching the root canal wall. The shear flow caused by acoustic microstreaming produces shear stresses along the root canal wall, which can remove debris and bacteria from the wall (Ahmad *et al.* 1988).

The influence of the insertion depth of an ultrasonically oscillating file on the ability to remove dentine debris from simulated canal irregularities (0.5, 2, 4, and 6 mm from the working length) in an extracted tooth model of a straight root canal and its influence on the flow of irrigant in both straight and curved canals was evaluated by Malki *et al.* (2012). Dye penetration and high-speed recordings of flow showed the static and dynamic behaviour of the flow during ultrasonic activation. The results of this study showed that the overall cleaning efficacy decreased with increasing distance between the file and the apex, with the depressions next to the file and within 3mm in front of the file being the cleanest. The flow observed from the visualization experiments matched this distance, suggesting a direct relation between flow and cleaning. The observed flow depth increased with increasing power setting; the curvature of the root canal had no influence on the flow depth. High-speed imaging showed a start-up phase with deeper fluid activation than in the steady phase afterward. The ultrasonically oscillating file could remove dentine debris up to 3 mm in front of the file tip, coinciding with the extent of the observed flow.

The generation and comparison of fluid movement induced by passive ultrasonic irrigantion (PUI) and photon-induced photoacoustic streaming (PIPS) was studied, using particle image velocimetry (PIV) and 6-µm melamine spheres in water (Koch *et al.* 2016). Measurement areas were 3-mm-long sections of the canal in the coronal, mid-root and apical regions of simulated root canals prepared to an apical size #30/0.04 taper. Fluid movement was analysed directly subjacent to the apical ends of ultrasonic insert or fibre optic tips as well as at mid-root and apically. During PUI, measured average velocities were around 0.03m/s in the immediate vicinity of the sides and tip of the ultrasonic file. Speeds decayed to non-measurable values at a distance of about 2mm from the sides and tip. During PIPS, typical average speeds were about ten times higher than those measured for PUI, and they were measured throughout the length of

the canal, at distances up to 20mm away. PIPS caused higher average fluid speeds when compared to PUI, both close and distant from the instrument. PIPS may be an alternative approach due to its ability to create high streaming velocities further away from the activation source compared to ultrasonic activation.

1.5.4.5 Cavitation and cavitational microstreaming

Cavitation in the fluid mechanical context can be described as the impulsive formation of cavities in a liquid through tensile forces induced by high-speed flows or flow gradients (Macedo *et al.* 2014). These bubbles expand and then rapidly collapse producing a focus of energy leading to intense sound and damage, e.g. pitting of ship propellers and pumps. Acoustic cavitation can be defined as the creation of new bubbles or the expansion, contraction and/or distortion of pre-existing bubbles (so-called nuclei) in a liquid, the process being coupled to acoustic energy (Leighton 1994).

According to Roy *et al.* (1994), two types of cavitation could occur during PUI of root canals: stable cavitation and transient cavitation. Stable cavitation could be defined as linear pulsation of gas-filled bodies in a low amplitude ultrasound field. Transient cavitation occurs when vapour bubbles undergo highly energetic pulsations. When the acoustic pressures are high enough, the bubbles can be inertially driven to a violent collapse, radiating shock waves and generating high internal gas pressures and temperatures. The energy at the collapse point is in some cases sufficient to dissociate the gas molecules in the bubble, which recombine radiatively to produce light, a process known as sonoluminescence (Crum 1994, Brenner *et al.* 2002). In the studies of Ahmad *et al.* (1988), Lumley *et al.* (1993) and Roy *et al.* (1994), sonoluminescence was used to detect transient cavitation.

The amount of cavitation generated around several endodontic instruments was measured by sonochemiluminescence dosimetry and visualized with long-exposure photography inside 4 root canal models of human dimensions and varying complexity (Macedo *et al.* 2014b). Cavitation was shown to occur in PUI at clinically relevant ultrasonic power settings in straight and curved canals, at the entrance of lateral canals and isthmuses, and also up to 2 mm beyond the tip of the file. No cavitation was detected around sonically oscillating instruments, driven at their highest

frequency. However, transient cavitation only occurs when the file can vibrate freely in the canal or when the file touches lightly (unintentionally) the canal wall (Lumley *et al.* 1993, Roy *et al.* 1994).

1.5.4.6 Continuous or Intermittent ultrasonic irrigation

Ultrasonic irrigation of the root canal can be performed with or without simultaneous ultrasonic instrumentation. Nowadays, the combination of simultaneous ultrasonic instrumentation and irrigation cannot be accepted as an alternative to conventional hand or rotary instrumentation (Abbott *et al.* 1991, Passarinho-Neto *et al.* 2006). This is due to reported operational difficulties to control intra-radicular dentine cutting, deviations in the shape of the canal such as ledges as well as strip perforations (Stock 1991, Lumley *et al.* 1992).

When canal shaping is performed without simultaneous ultrasonic instrumentation, but only irrigant agitation, the term passive ultrasonic irrigation (PUI) can be used to describe the technique. The term PUI was first used by Weller *et al.* (1980) to describe an irrigation scenario where there was no instrumentation, planing, or contact of the canal walls with an endodontic file or instrument (Jensen *et al.* 1999). With this noncutting technology, the potential to create aberrant shapes within the root canal was reduced.

PUI can be performed with a small file or smooth wire (size 10–20) oscillating freely in the root canal to induce powerful acoustic microstreaming. PUI can be an important supplement for cleaning the root canal system and, compared with traditional syringe irrigation, it removes more organic tissue, planktonic bacteria and dentine debris from the root canal and it is more advantageous to apply ultrasonics after completion of canal preparation (van der Sluis *et al.* 2007).

Despite the fact that intentional contact between the ultrasonic file and the root canal wall is not currently recommended during PUI, unintentional contact may occur due to the dimensions and complex geometry of the root canal system (Vertucci 2005). A laboratory study was conducted in order to quantify in a simulated root canal model the file-to-wall contact during ultrasonic activation of an irrigant (Boutsioukis *et al.* 2013). Inadvertent file-to-wall contact

occurred in all cases during 20% of the activation time and file oscillation was affected by contact during 94% of the activation time. However, the file oscillation was not dampened completely due to the contact and hydrodynamic cavitation was detected. Therefore, the authors proposed that the term ‘Passive Ultrasonic Irrigation’ should be amended to ‘Ultrasonically Activated Irrigation’ (UAI). This new definition will be applied to the entire text as it represents better the agitation procedure, which clearly is not passive, since tips may bind on root canal axial walls.

Despite the use on non-cutting ultrasonic tips, the results of two studies disclosed with the aid of micro-computed tomography that UAI may result in uncontrolled removal of dentine in straight and curved root canals, at manufacturer-recommended power settings (Boutsioukis & Tzimpoulas 2016, Retsas *et al.* 2016). Scans showed that the dentine defects had a maximum varying depth between 0.07 and 0.09mm in straight canals, whereas a maximum depth of 0.18 mm was identified in curved canals (Boutsioukis & Tzimpoulas 2016, Retsas *et al.* 2016).

Two flushing methods might be used during PUI, namely a continuous flush of irrigant from an ultrasonic handpiece or an intermittent flush technique by using syringe delivery (Cameron 1988). A needle-holding adapter to an ultrasonic handpiece has been developed by Nusstein (2005) (ProUltra PiezoFlow, Dentsply Sirona, USA) (Figure 1-11). During ultrasonic activation, a 25-gauge irrigation needle is used instead of an endosonic file. The unique feature of this needle-holding adapter is that the needle is simultaneously activated by the ultrasonic handpiece, while an irrigant is delivered from an intravenous tubing connected via a luer-lock to an irrigation-delivering syringe. The irrigant can thus be delivered apically through the needle under a continuous flow.

Figure 1-11 The ProUltra PiezoFlow system for continuous ultrasonic irrigation (adopted by www.dentsplysirona.com).

In the intermittent flush technique, the irrigant is injected into the root canal by a syringe and replenished several times after each ultrasonic activation cycle. The amount of irrigant flowing through the apical region of the canal can be controlled because both the depth of syringe penetration and the volume of irrigant administered are known. This is not possible with the use

of the continuous flush regime. Both flushing methods have been shown to be equally effective in removing dentin debris from the root canal in an ex vivo model when the irrigation time was set at 3min (van der Sluis *et al.* 2006). Most of the existing studies evaluated the effectiveness of UAI by using the intermittent flush technique, therefore any further references in literature will describe the intermittent flush UAI.

After shaping the root canal, cleaning and debridement can be completed with UAI or a final flush of syringe irrigation. Remaining debris forming from the cutting instruments may contain bacteria and obstruct the flow of irrigating solutions into lateral canal anatomy. Conventional syringe irrigation may allow good irrigant control, however it remains questionable whether it is effective in flushing out tissue remnants and cleaning the most apical portions of the root canal system (Thomas *et al.* 2014).

Traditionally, debridement of root canals has been evaluated by means of sectioning methods combined with SEM (Urban *et al.* 2017) and examination of cleanliness of artificially prepared apical grooves packed with dentine debris (van der Sluis *et al.* 2006, De Moor *et al.* 2010, Haapasalo *et al.* 2014). In such studies, the use of qualitative scoring by clinicians and the performance of the investigations on 2-dimensional cross-sections of teeth give a limited view of where debris is accumulating. Moreover, the process of cross-sectioning may alter the location of debris and this problem is further compounded by the complex anatomy in mandibular molars.

Hard-tissue debris accumulated after different chemomechanical preparation protocols has also been quantified using micro-CT in isthmus-containing mesial roots of mandibular molars (Paque *et al.* 2009, 2011, Robinson *et al.* 2012, Freire *et al.* 2015). From the studies where UAI and conventional syringe irrigation were compared, it can be concluded that NaOCl combined with UAI is more effective in removing debris and the forming smear layer (Freire *et al.* 2015, Leoni *et al.* 2017, Verstraeten *et al.* 2017). Additionally, the application of UAI presented similar effectiveness in root canal debridement with other agitation systems, such as EndoVac System (Freire *et al.* 2015), XP Endo Finisher (XPEF) (Leoni *et al.* 2017) and PIPS (Verstraeten *et al.* 2017).

1.6 Application of XP Endo Finisher (XPEF)

Recently, the XPEF was introduced as a new type of anatomical finishing file as a final step in improving root canal cleaning. XP Endo Finisher (XPEF) (FKG Dentaire, Switzerland) is a new file that has been recently introduced as a final disinfection step to disturb the bacterial biofilm. Irregularities of the canal system create difficulties for clinicians during root canal treatment. The manufacturer of XP-endo Finisher recommends its use for the chemomechanical preparation of root canals with highly complex morphologies such as internal root resorption cavities (FKG 2015).

It is a 25/.00 instrument produced using a special type of alloy, the NiTi MaxWire (Martensite-Austenite Electropolish-FleX, FKG) (Trope & Debelian 2015). According to the manufacturer, the file is straight in its M phase when it is cooled, and it will change into A phase when it is exposed to body temperature where it will acquire its unique spoon shape with a length of 10 mm from the tip and a depth of 1.5 mm because of its molecular memory (Figure 1-12) (Trope & Debelian 2015, FKG Dentaire SA 2016). So far, there is no available data on the kinetics of the XPEF and its mode of agitation, when the root canal is filled with irrigant.

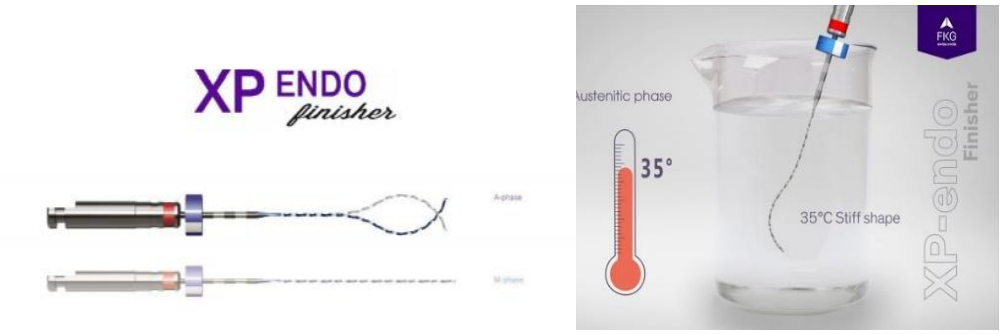


Figure 1-12 The XP Endo Finisher file and its transition from martensitic to austenitic phase in body temperature.

The application of XPEF for the removal of hard-tissue debris from the mesial root system of mandibular molars, inter-appointment pastes such as calcium hydroxide and poly-antibiotic pastes and root filling remnants presented similar favourable results to different methods of irrigant agitation (Gokturk *et al.* 2016, Leoni *et al.* 2017, Silva et al. 2017, Wigler *et al.* 2017). In addition, the results of several in vitro studies showed that XPEF is a satisfactory adjunct in enhancing a disinfection regime against developed biofilms in dentine grooves and dentinal

tubules, as well as removing soft tissues from intracanal lateral grooves or artificial root resorption cavities (Azim *et al.* 2016, Alves *et al.* 2016, Estevez *et al.* 2017).

1.7 Irrigant penetration in lateral canals

In single-rooted teeth, lateral canals branch from the main, with diameters ranging from a minimum of 10 up to 400μm (Miyashita *et al.* 1997). Such narrow orifices create a surface tension barrier that does not allow adequate mixing between the irrigant and the liquid within the canal. The study of irrigant penetration in lateral canals has gained significant attention mostly in relevance with root canal obturation materials and techniques (Fernández *et al.* 2016). Histologic sections of extracted teeth have revealed that the lateral canals are not completely cleaned, and, when filled with a root filling material, they also contain vital or necrotic pulp tissue and bacteria (Riccucci & Siqueira 2011).

Artificial lateral canals with varying diameters of more than 0.12mm have been drilled in decalcified natural teeth (Smith *et al.* 2000) or resin blocks (Dulac *et al.*1999, Silver *et al.*1999). In addition, artificial lateral canals with internal diameters varying from 0.25mm to 0.3mm were constructed in a transparent solidified polydimethylsiloxane model (Macedo *et al.* 2014) and 3-D printed transparent root blocks (Mohmmed *et al.* 2018), respectively.

De Gregorio *et al.* (2010) evaluated the effect of currently used irrigation and activation systems on the penetration of NaOCl into simulated lateral canals and to working length in a closed system. The collected single rooted specimens were chemomechanically prepared, cleared and lateral canals were created by inserting 06 K-files at 2, 4.5 and 6 mm of working length crossing from the buccal to the lingual walls, perpendicularly to the external surface. To resemble the clinical situation, a closed system was created by coating each root with soft modelling wax. The contrast solution used as main irrigant contained 90% of 5.25% NaOCl and 10% Kuraray caries detector solution. Irrigation was performed with a 30-G side-vented needle and a total volume of 1.5ml contrast solution was delivered 2mm short of working length. The samples were evaluated by direct observation of the images recorded under the dental operating microscope. The results demonstrated that the application of apical negative pressure (ANP) was

superior at reaching working length, and UAI was the most effective at lateral canal penetration. ANP demonstrated limited activation of the irrigant in non-instrumented areas, represented by lateral canals. This limitation could be explained by the osmotic drawing effect described by Pashley *et al.* (1985). The addition of EDTA in a previous study did not result in better penetration of irrigants into the lateral canals (de Gregorio *et al.* 2009). In a similar experimental protocol the same research group examined the ability of NaOCl to penetrate simulated lateral canals and to reach working length using the self-adjusting file (SAF). The pecking motion of SAF system allowed for further penetration of the irrigant compared to conventional syringe irrigation, but failed to irrigate at working length as well as within the full length of simulated lateral canals (de Gregorio *et al.* 2012).

Castelo-Baz *et al.* (2012) compared the effects of 2 ultrasonic irrigation techniques (passive; PUI and continuous; CUI) on the penetration of NaOCl into the main canal and simulated lateral canals of extracted teeth. The authors adopted similar methodology to De Gregorio *et al.* (2010) with regard to specimen and lateral canal preparation as well as recording and observation of the irrigated canals under magnification. To examine irrigating solution penetration, 20% Chinese ink (Sanford Rotring GmbH, Hamburg, Germany) was added to a 5% NaOCl solution and delivered into the root canals. The results showed a significantly higher penetration of irrigant into the lateral canals in the CUI group. PUI and CUI did not differ significantly in solution penetration into the apical thirds of the main canals. Conventional irrigation showed a significantly lower penetration of NaOCl into the main and lateral canals compared with the CUI and PUI groups.

Spoorthy *et al.* (2013) evaluated the effect of conventional syringe irrigation, an ANP system, an UAI system and a combination of both apical ANP and passive UAI on the penetration of the irrigating contrast solution up to working length and into simulated lateral canals. The root canals of single-rooted teeth were initially instrumented and irrigated. On completion of the shaping procedures, teeth were cleared and simulated lateral canals were prepared using techniques described previously (Robertson & Leeb 1982, de Gregorio *et al.* 2009). In each specimen, three simulated lateral canals were created at 2-, 4- and 6-mm levels from the root apex using a 06-size C+ file (Dentsply Maillefer, Ballaigues, Switzerland). The contrast solution was prepared by mixing 40% Indian ink with 60% of NaOCl 5% in the ratio of 2:3 and delivered

into the root canals (Castelo-Baz *et al.* 2012). The assessment of the samples was performed by direct observation of the images taken using a digital camera. The depth of penetration of the contrast solution up to the working length and into the simulated lateral canals was analysed with a previously proposed scoring system, depending on the penetration level (50% of lateral canal length) (de Gregorio & Estevez 2010). The combination (ANP and UAI) and the UAI group exhibited significantly greater irrigant penetration into lateral canals at the 6- mm level. At the 4- and 2- mm levels, the combination (ANP and UAI) had significantly greater irrigant penetration into the lateral canals than the other groups.

The application of CFD has been well documented previously for the examination of irrigant flow according to syringe type, flow rate, taper, needle insertion depth. However, according to Wang *et al.* (2015) the applied model was based on a relatively simple anatomy of a single root canal. There have been no studies on the effectiveness of irrigation in complex root canal systems. The aim of their study was to investigate the effect of the orientation of a side-vented needle on the irrigant flow pattern in a C-shaped root canal system using CFD and to evaluate the real-time replacement of irrigant in the existing lateral canal. A mandibular second molar with a complete C-shaped canal system was chosen, prepared, scanned and the images were exported to design software. A 30-G, side-vented irrigation needle was positioned with the open notch facing the lateral canal (A) and rotated 90^{o} (B), 180^{o} (C), and 270^{o} (D) clockwise. The flow pattern, irrigant replacement, velocity distribution, wall stress distribution, and apical pressure in the canal were analyzed. Most of the irrigant flowed to the canal outlet from the adjacent instrumented canal space with different trajectories in the 4 cases. The concentration of scalar immediately below the tip of the needle was exchanged quickly; the length of the cleared zone extended 3mm beyond the tip of the needles. The depth of circulation in the lateral canal in all cases increased during the first 0.2s. After that, the exchange of irrigant reached a stable phase. Irrigant penetration in the lateral canal was greatest in positions A and B (1-1.2mm) reaching almost half length of the lateral canal. The results of the exchange of irrigant in the main canal show that the length of the cleared zone extended 3mm beyond the tip of the needles, which was in accordance with previous studies in models with simple root canal anatomy (Shen *et al.* 2010).

1.8 Irrigant penetration in dentine tubules

Studies on the microbiology of endodontic infections have clearly demonstrated that the bacteria present in the necrotic root canal system can be found in the main root canal space, lateral canals, and dentinal tubules (Ørstavik *et al.* 1990). According to literature, the number of infected tubules and the depth of penetration of bacteria are highly variable, ranging from 150mm to half the distance between the main root canal and the cementodentinal junction (Ando & Hoshino 1990). Whilst the effectiveness of irrigation has been studied in the main root canal (van der Sluis *et al.* 2005), little is known about the penetration into dentinal tubules of endodontic irrigants, including NaOCl.

A prerequisite to any disinfectant effect in dentine is that the agent penetrates into the dentinal tubules. Therefore, it is surprising how little is known about the penetration of irrigating solutions into dentine. The reason for this is probably the technical and chemical difficulty in measuring such small volumes at exact distances. In addition, when dentine is cut or fractured, this in itself is likely to have a major effect on liquid movement in dentinal tubules, making the measurements unreliable.

In 2010, Zou *et al.* published a study describing the penetration of NaOCl into dentine using a dye model. The collected root specimens were initially prepared and then sectioned perpendicular to the long axis. The crowns and apical thirds of all the teeth were removed. The remaining roots were processed into 4-mm-long blocks and stained overnight in crystal violet dye solution. The stained blocks were treated by 1%, 2%, 4%, and 6% NaOCl for 2, 5 and 20min at 20^0C, 37^0C and 45^0C, respectively. The depth of penetration of NaOCl was determined by bleaching of the stain. After the exposure, the NaOCl was washed away with running water and the blocks were blotted dry and fractured, allowing direct observation and measurement of the bleached area under a light microscope at magnifications of 20x and 40x. A short exposure time and low concentration of NaOCl (1%; 2min) led to short penetration distances (77μm), whilst higher concentration and increased exposure time (6%; 20min, 45^0C) led to penetration depths up to 300μm (Zou *et al.* 2010). Temperature had a modest effect within each group on the depth of penetration and overall within each time group, depth of penetration with 1% NaOCl was about 50%–80% of the values obtained with 6% NaOCl (Zou *et al.* 2010).

In a similar study, the effect of dentine pretreatment with 17% EDTA, 1% citric acid, and 1% peracetic acid associated with 2.5% NaOCl penetration into dentinal tubules was evaluated (Kuga *et al.* 2011). Instrumented root fragments were subjected to 2 consecutive 10-min immersion periods in one of the previous acid solutions combined with 2.5% NaOCl. The analysis of NaOCl penetration was similarly performed by measuring the depth of bleached crystal violet stain under 50x magnification. The results showed a low penetration using 2.5% NaOCl either alone or mixed with EDTA, citric acid, or peracetic acid. For NaOCl only, the depth of penetration was 107mm. Interestingly, mixing with EDTA greatly reduced the depth of bleaching, while mixing with peracetic acid seemed to increase the penetration, although due to the small sample size the difference was not statistically significant. The authors highlighted though, that bleaching of the stained dentin does not necessarily correlate well with the ability of the solutions to kill bacteria in the same area (Kuga *et al.* 2011).

Akcay *et al.* (2017) evaluated the effect of different agitation techniques on NaOCl penetration into dentinal tubules, by using a confocal laser scanning microscope (CLSM). In this study, extracted single-rooted human mandibular premolars were instrumented up to size 40 and randomly divided into 5 groups, based on the activation technique of the final irrigation solution as follows: conventional irrigation (control group), sonic activation, UAI, PIPS activation, and Er:YAG-Preciso tip activation. In each group, 5ml of 5% NaOCl labeled with fluorescent dye was used during the activation as the final irrigation solution. Specimens were sectioned at 2.5 and 8 mm from the apex and then examined under a confocal microscope to calculate the dentinal tubule penetration area. Both Er:YAG laser (Preciso/PIPS) activations exhibited a significantly higher penetration area than the other groups. Additionally, UAI had significantly higher penetration than the sonic activation group and the control group. Statistically significant differences were also found between each root canal third (coronal > middle > apical). The results from the present study supported the use of Er:YAG laser activation (Preciso/PIPS) to improve the effectiveness of the final irrigation procedure by increasing the irrigant penetration area into the dentinal tubules (Akcay *et al.* 2017). The activation of the irrigant and the creation of the streaming with the Er:YAG laser had a positive effect on the irrigant penetration.

1.9 Interaction of NaOCl with planktonic bacteria

NaOCl is a strong, non-specific oxidising disinfectant and has high effectiveness against planktonic populations of microbial cells. Several laboratory studies have been proposed for the examination of the effectiveness of NaOCl against planktonic bacteria including agar diffusion tests, broth dilution tests and extracted tooth models (Park *et al.* 2013).

Agar diffusion and broth dilution tests, are generally considered as *in vitro* direct contact tests. In agar diffusion tests, various types of single endodontic pathogens are grown into individual agar plates supplemented with nutrient media. The diameters of the growth inhibition zones produced by the tested disinfectants, at different concentrations and observation periods, are measured to produce comparative outcomes (Siqueira *et al.* 1998). In broth dilution tests, endodontic pathogens are initially grown and suspended in a liquid medium, which is mixed with the tested disinfectants. The timing for irrigants to kill all microbial cells is recorded and is considered as a measure of antimicrobial efficiency (Gomes *et al.* 2001). As a general concluding remark, the antimicrobial action of the tested irrigants was related to type, concentration, time of interaction as well as the microbial susceptibility (Vianna *et al.* 2004, Ferraz *et al.* 2007, Tirali *et al.* 2013). However, they do not represent the conditions that exist in an infected root canal and the outcomes of such laboratory studies are generally over-estimated, whilst they have limited value in translational research (Pappen *et al.* 2010).

The initial application of extracted tooth models was considered to be more informative, but several limitations exist with regard to the control of the growth of bacterial species in planktonic forms or the lack of justification of biofilm formation (Shen *et al.* 2013).

Siqueira *et al.* (1997) examined the effectiveness of 4% NaOCl with three irrigation methods in the elimination of E. faecalis from the root canal *in vitro*. The infected root canals were treated with irrigation with 2ml NaOCl and were agitated with hand files and ultrasonics, or irrigated alternated with hydrogen peroxide. The use of sterile saline solution served as the control. Bacterial sampling with paper points was used and the appearance of broth turbidity was indicative of bacteria remaining in the root canal.

Vivacqua-Gomes *et al.* (2005) assessed the presence of *E. faecalis* after root canal treatment in single or multiple visits in an ex vivo model, comprised of human extracted premolar teeth infected ex vivo with *E. faecalis* for 60 days. The canals were then prepared using a crown-down technique with System GT and Gates-Glidden burs and irrigated with 2% CHX gel. The specimens were divided into five groups (G1, G2, G3, G4 and G5) according to the time elapsed between chemical-mechanical preparation and root canal filling, the irrigant solution used and the use or no use of a calcium hydroxide intra-canal medicament. The teeth were then root-filled and incubated for 60 days at 37^0 C. Dentine chips were removed from the canal walls with sequential sterile round burs at low speed. The samples obtained with each bur were immediately collected in separate test tubes containing brain heart infusion (BHI). The samples were placed onto agar plates and colony forming units (CFU) were counted after 24h. *E. faecalis* was recovered from 20% (3/15 specimens) of G1 (CHX irrigation and immediate root filling in a single visit), 25% (4/15 specimens) of G2 (CHX irrigation and filling after 14 days use of a calcium hydroxide dressing in multiple visits), 40% (2/5 specimens) of G3 (CHX irrigation and filling after 7 days), 60% (3/5 specimens) of G4 (saline irrigation and filling after 7 days) and from 100% (5/5 specimens) of G5 (saline irrigation and immediate filling without sealer). Neither single- nor multiple-visit root canal treatment ex vivo, eliminated *E. faecalis* completely from dentinal tubules. Up to 60 days after root filling, E. faecalis remained viable inside dentinal tubules. When no sealer was used, E*. faecalis* presented a higher growth rate. NaOCl was not used in this study, therefore, the results should be interpreted with caution.

A study reported the effect of three different concentrations of NaOCl (of 0.5%, 2.5% and 5.25%) both using hand rotary instrumentation techniques on artificially infected dentine with *E faecalis*. The examination at three depths showed that the highest concentration of sodium hypochlorite was most effective (Berber *et al.* 2006). In a similar study, Câmara *et al.* (2009) assessed the the antimicrobial activity of 0.5%, 1% and 2.5% NaOCl, at each point of instrument change of the ProTaper Universal system, in human extracted mandibular premolars with single root canals. The microorganisms were eliminated after the instrumentation with S1 file in all tested irrigants, except for 1 sample in S1 at 0.5% NaOCl which showed positive growth.

Brito *et al.* (2009) compared the intracanal bacterial reduction promoted by chemomechanical preparation with 3 different irrigation techniques. The root canals of human

extracted teeth were contaminated with *E. faecalis* ATCC 29212 for 7 days and then randomly distributed into 3 experimental groups (group 1, conventional irrigation with NaviTip needles inserted up to 3 mm short of the working length; group 2, same as group 1, supplemented with final irrigant activation by the EndoActivator system; group 3, irrigation with the EndoVac system). NaOCl and EDTA were the irrigants used in all experimental groups. The overall preparation time was kept constant for the groups, but the total volume ranged from 20ml (groups 1 and 2) to 43 ml (group 3). The control group was irrigated with saline solution. Samples taken before and after chemomechanical procedures were cultured, and the CFU were counted. The 3 experimental groups treated with NaOCl and EDTA were significantly more effective than the control group with saline in reducing CFU counts. There were no significant differences between the 3 techniques tested.

In the previous studies, sampling was performed with paper points, or by drilling dentine chips from the root canal walls. The CFU measurement provides information on the number of viable bacteria, which are possible to be collected in the sampling process. However, commonly used sampling methods are best suited for planktonic bacteria and those bacteria that are loosely attached to dentine walls. Paper points can only reach areas where endodontic files used for instrumentation are able to go. Untouched areas will also be left untouched by the sample collecting instrument. Sampling with paper points is unlikely to effectively collect bacteria from difficult to reach areas such as dentinal tubules, isthmuses, anastomoses. Therefore, culturing from the root canal is also a sensitive and technically time-consuming method, which may get further complicated by several confounding factors. If the differences in killing are not great, inherent variations due to the method make it difficult to obtain statistically significant differences. With the advent of the biofilm concept, the much greater resistance of bacterial strains in biofilms compared with their planktonic, “free-floating” counterparts (Costerton *et al.* 1994, Johnson *et al.* 2002) raises concerns about the validity of laboratory studies using only liquid-grown cultures.

1.10 Bacterial growth in biofilms

Microorganisms are widespread in nature, and many can adhere to surfaces, forming biofilms, which can be very damaging to human health and medical applications (Zheng *et al.* 2018). In medical microbiology, the importance emanates from estimates, which indicate that biofilm infections are responsible for 65–80% of human infections in the developed world (Costerton 2004). It has also been long known that biofilm resistance to antibiotics increases significantly when bacteria form biofilms (Gristina *et al.* 1987, Evans & Holmes 1987, Sedlacek & Walker 2007) andmaturity of the biofilm has been shown to play a role in resistance, resulting in a lesser effect of antibiotic treatment than in young biofilms (Anwar *et al.* 1992). Antibiotics are often applied to keep bacterial and biofilm infections at bay, however, studies suggest that antibiotic treatment, especially when the doses are insufficient, can lead to a shift in the microbiota and increase biofilm and extracellular matrix formation (Bleich *et al.* 2015, Mart *et al.* 2014).

Biofilms can be defined as a sessile multi-cellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substances (EPS) (Donlan & Costerton 2002, Fleming & Wingender 2010). Bacterial communities can anchor on implant or tissue surfaces and produce biofilms, which are commonly surrounded by the EPS matrix (Flemming *et al.* 2007, Boisvert *et al.* 2016, Archiola *et al.* 2018, Beam & Osmon 2018). A microbial biofilm can consist of multiple single cell organisms of a single species of bacteria. More commonly, however, it consists of multiple, co-dependent, interacting species (Davey *et al.* 2000, Hall-Stoodley *et al.* 2004).

Broadly speaking biofilm development is the transition of planktonic microbes into a resistant mature biofilm, surrounded by an extracellular matrix. The processes and environmental factors for biofilm formation vary depending on species, however, the developmental steps from initial attachment to a surface, formation of microcolonies, and maturation into an EPS matrix covered biofilm, followed by dispersion are steps that generally apply (Davey *et al.* 2000).

The initial attachment of biofilms and subsequent biofilm formation occurs when microbes encounter conditions favourable for the transition from a planktonic state to surface attached colonisation. It is important to note that favourable conditions and mechanisms of attachment vary from species to species (Toole *et al.* 2000). Surfaces exposed to growth medium or human body

fluids form an initial film of minerals, proteins and polysaccharides. The type of the initial film and the involved microbial strains may affect the increase or inhibition of biofilm formation. A recent study showed that increased biofilm formation may occur on rougher surfaces as well as on hydrophobic surfaces, which favour initial protein aggregation and thus induce biofilm growth (Lüdecke *et al.* 2014).

The attachment of the microbial strains to a surface is the first step leading to maturation of the biofilm, and, to the upregulation of factors in favour of life as a sessile colony (Kostakioti *et al.* 2013). The formation of the EPS is another main shift in the transition of a mature biofilm into a robust biofilm structure (Flemming & Wingender 2010). The EPS matrix is a fundamental part of the biofilm nature and can make up over 90% of the dry mass in a biofilm. The EPS matrix consists of various polymers, excreted by the microbes within the biofilm themselves, predominantly including the following:

- Exopolysaccharides: Being the main component of the matrix, they are long linear or branched molecules involved in initial surface attachment, biofilm formation as well as structure (Colvin *et al.* 2011).

- Proteins: They are also a large component of the EPS, being involved in enzymatic digestion of nutrients within the biofilm and rearrangement of the EPS matrix structure. They can further facilitate dispersion of bacterial cells, often in response to a change in nutrient availability. Non-enzymatic proteins such as lectins, which bind to carbohydrates are involved in formation, crosslinking and stabilisation of the biofilm (Toyofuku *et al.* 2012).

- Matrix DNA: It has been shown to function as an adhesion as well as for intercellular connection (Harmsen *et al.* 2010, Flemming & Wingender 2010)

1.10.1 The role of EPS matrix

The role of EPS matrix can be summarised to the following:

(i) EPS mediates biofilm adhesion to surfaces, very often acting as a “biological glue”.

(ii) EPS also provides mechanical stability to the biofilm and allows for extracellular enzymes to accumulate and exert important activities, which include nutrient acquisition and co-operative degradation of complex macromolecules.

(iii) It keeps biofilm cells in close proximity, thus allowing for interactions including quorum sensing, genetic exchanges, and pathogenic synergism.

(iv) In periods of nutrient deprivation, it can serve as a nutrient source, although some components of the matrix may be only slowly or partially degradable.

(v) It retains water and maintains a highly hydrated microenvironment surrounding the biofilm populations.

(vi) It plays a protective role against host defense cells and molecules as well as antimicrobial agents (Flemming & Wingender 2010).

Moreover, largely due to EPS production, biofilm formation can protect its community from other chemical disinfection, the host immune response, germicides and even from physical removal, as biofilm structures can offer resistance to mechanical disruption when grown in challenging environments (Donlan & Costerton 2002). EPS production and other defence mechanisms are often increased when the biofilm community is confronted with unfavourable environmental conditions, such as starvation or toxic stress (Donlan & Costerton 2002, Fux *et al.* 2005).

An important part of biofilm survival is the ability to adapt to a large range of environments, including changes in availability of nutrients, change in pH and even presence of antimicrobial agents. Biofilm communities can trigger a stress response which can lead to physiological changes resulting in a biofilm phenotype more suited to the change in environment (Fux *et al.* 2005, Poole 2012). Dormancy has also been shown as a response to various unfavourable environments, such as nutritional or chemical stress, extreme temperatures and oxygen availability. Entering such a dormant and non-culturable state can also result in such bacteria remaining undetected (Li *et al.* 2014). These are some of many means of biofilms to adapt to hostile environments, including their capacity to adapt as a result of the multispecies nature and

interaction, as well as inherent bacterial survival mechanisms, which justify their prevalence during infections.

1.10.2 Biofilm formation in infected root canals

It has been well documented that bacterial growth within a necrotic root canal is the main cause of apical periodontitis (Kakehashi *et al.* 1965, Möller *et al.* 1981). If bacterial invasion within the root canal is not arrested or removed, inflammation and subsequent necrosis of the pulp tissue will inevitably occur. Once the tissue is necrotic, the progression of the infection in the entire root canal space occurs and the host defence retracts to the periapical tissue (Zehnder & Belibasakis 2015). Periapical disease normally involves an inflammatory lesion around the root apex and can presents itself as an acute or chronic inflammation. This lesion is the site of bacterial interaction with the host immune response. A number of host cells are recruited to the site of inflammation including polymorphonuclear leukocytes, macrophages, eosinophils, plasma cells, mast cells and epithelial cells (Abbott 2004, Zehnder & Belibasakis 2015).

Root canal infections have a different nature than that of caries or periodontitis because they become established in originally sterile compartments of the oral cavity. From an ecological perspective, the root canal can be considered a highly controlled environment with a limited number of niches. The main limiting factors in root canal niches that influence bacterial colonization are, for instance, oxygen and nutrient availability (Auschill *et al.* 2001). The root canal system is a very selective environment, due to limited amount of oxygen and nutrients, making it uninhabitable for most microbial species (Chávez de Paz 2007).

Biofilm formation in root canals was firstly hypothesized by Svensäter & Bergenholtz (2004). It is probably initiated at some time after the first invasion of the pulp chamber by planktonic oral organisms following dentine tissue breakdown and/or pulp exposure. At this point, the developing inflammatory lesion will provide the fluid vehicle for the invading planktonic organisms to multiply and attach to the root canal walls. While inflammation moves successively toward the apex, bacteria may detach from inner root canal surfaces and mass in the inflammatory lesion per se (Siqueira *et al.* 2012). This observation explains how the progressing inflammatory

lesion serves as a fluid source for bacterial biofilm detachment and colonization of other inaccessible sites in the root canal.

The evidence that apical periodontitis is a biofilm induced disease comes from *in situ* investigations using histological sections, optical microscopy, electron microscopy or combined confocal-fish microscopy (Nair 1987, Molven *et al.* 1991, Siqueira *et al.* 2002, Ricucci *et al.* 2009, Carr *et al.* 2009, Schaudinn *et al.* 2009). These studies showed the colonization of the root canal system in primary or persistent/secondary infections by bacteria organised in sessile biofilm-like structures, covering the dentinal root canal walls. Apical ramifications, lateral canals, and isthmuses connecting main root canals have all been shown to harbor bacterial cells frequently organized in biofilms (Nair *et al.* 2005, Ricucci & Siqueira 2008, Ricucci & Siqueira 2010). Moreover, biofilms adhered to the external apical root surface (extraradicular biofilms) have been regarded as a possible cause of post-treatment apical periodontitis (Tronstad *et al.* 1990, Ferreira *et al.* 2004, Ricucci *et al.* 2005). Thus, when biofilms are formed on surfaces located beyond the reach of mechanical removal and the effects of antimicrobials, host-derived proteins from remaining necrotic tissues and bacterially produced adhesive substances will provide the proper prerequisites for the survival of microbes.

Although the concept of apical periodontitis as a biofilm-induced disease has been built upon these observations, the prevalence of biofilms and their association with diverse presentations of apical periodontitis were only very recently investigated. Ricucci & Siqueira (2010) evaluated the prevalence of biofilms in untreated teeth (primary infections) and treated teeth (persistent/secondary infections) with apical periodontitis and looked for associations between endodontic biofilms and clinical/histopathological conditions. The authors reported that intraradicular biofilm arrangements were in general observed in the apical segment of 77% of the root canals of teeth with apical periodontitis (80% in untreated canals and 74% in treated canals). In some cases, the biofilm even formed on the inflamed soft tissue near the apical foramen. The observed bacterial biofilms were morphologically thick and composed of several layers of bacterial cells. The biofilms also invaded dentinal tubules underneath the bottom of the biofilm structure. Bacteria were also seen in the lumen of the main canal, ramifications, and isthmuses as flocs and planktonic cells, either intermixed with necrotic pulp tissue or possibly suspended in a fluid phase. Bacterial flocs in clinical specimens may originate from the growth of cell

aggregates/co-aggregates in a fluid or they may have detached from biofilms (Hall-Stoodley & Stoodley 2009). Along with planktonic bacterial cells, flocs may play a role in the pathogenesis of acute clinical forms of apical periodontitis (Siqueira & Rôças 2009). Extraradicular biofilms were very infrequent, being observed in only 6% of the cases. The prevalences of intraradicular biofilms in teeth associated with apical cysts, abscesses, and granulomas were 95%, 83%, and 69.5%, respectively. Biofilms were significantly associated with epithelialized lesions.

1.11 Interaction of NaOCl with endodontic biofilms

During the last decade, the research interest in endodontic biofilms has grown rapidly and several studies have measured the effectiveness of different disinfecting agents against biofilms. Significant variability and lack of standardisation of irrigation parameters such as concentration, time of exposure as well diversities in microbial growth phase, biofilm models and type of substrates is noticeable.

Several substrates have been used for the growth of single- or multi- species biofilms, including membrane filter disks (Spratt *et al.* 2001), 24-well polystyrene tubes (Ozok *et al.* 2007), cellulose nitrate membranes (Giardino *et al.* 2007), nitrocellulose filter membranes (Bryce *et al.* 2009), glass (Williamson *et al.* 2009), flow cell system (Dunavant *et al.* 2006), hydroxyapatite (HA) disks (Niazi *et al.* 2014), HA disks coated with collagen type I (Shen *et al.* 2010), root sections (Clegg *et al.* 2006) and dentine blocks (Liu *et al.* 2010).

Biofilm experiments conducted on polymer, plastic polycarbonate or glass substrate may not provide a true indication of the bacteria–substrate interaction. A general consensus from these study models was that the effectiveness of the applied disinfecting agents depended on the type of the organism in the biofilm, the growth pattern in monospecies or dual species, the contact time and the maturity of the grown biofilm (Spratt *et al.* 2001, Ozok *et al.* 2007, Williamson *et al.* 2009). These parameters should be taken into consideration, when new biofilm studies are designed.

1.11.1 Biofilm growth in HA disks

The use of HA disks (Niazi et al. 2014) or HA disks coated with type I collagen (Shen *et al.* 2009) may provide an acceptable substrate for multi-species biofilm growth due to chemical similarity with the inorganic phase of dentine. Niazi *et al.* (2014) characterised and developed nutrient-stressed biofilms, which were grown on hydroxyapatite discs and proposed the use of enzymic irrigation for the disruption of biofilms. The use of CLSM showed that nutrient starvation reduced the number of viable bacteria at the biofilm surface level and trypsin and proteinase K were effective in killing bacteria and disrupting the biofilm.

Shen *et al.* (2011) examined the susceptibility of multispecies biofilms to root canal irrigants, at different phases of growth. The multispecies biofilms were grown from plaque bacteria on collagen-coated hydroxyapatite discs in brain-heart infusion broth for time periods ranging from 2 days to several months. Biofilms of different age were subjected to 1-, 3-, or 10-min exposure to 2% CHX or CHX-Plus. After treatment, the volume ratio of dead bacteria in biofilms was assessed by CLSM and SEM. An increase in the thickness of biofilms was noticed in a period between 2 days and 3 weeks. A steady state was then reached under nutrient-limiting conditions, up to 12 weeks of observation. Mature biofilms were more resistant compared to young biofilms after treatment with both CHX solutions. The resistance of mature biofilms under the nutrient-limiting phase (6-12 weeks) remained stable and was similar to 3-week-old biofilm. The results emphasized the importance of standardization of factors such as biofilm age, when studying the effectiveness of disinfecting agents against biofilm bacteria. However, this model does not simulate the fine details of dentine microanatomy due to the absence of dentinal tubules. But the standard shape of the disks allowed for reproducible biofilm growth with consistent characteristics.

Stojicic *et al.* (2013) assessed the effect of the source of biofilm bacteria, the level of biofilm maturation and the type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents. Multispecies biofilms from plaque bacteria of 6 donors were grown for up to 8 weeks on collagen-coated HA disks. After 1, 2, 3, 4, and 8 weeks of growth the biofilms were exposed to 1% NaOCl, 0.2/0.4% iodine-potassium iodide and 2% CHX for 1 and 3 minutes. The percentage of killed biofilm bacteria was determined by using CLSM. The study confirmed the

findings of Shen *et al.* (2011), as 1- and 2-week-old biofilms were moderately or very sensitive to the tested disinfecting agents, which killed 20%-100% of the biofilm bacteria. After 3 weeks of growth the biofilm bacteria were more resistant to the same agents, and only 10%-30% of the bacteria were killed. The same pattern of the effect of biofilm age was observed in all 6 biofilms and with all 3 disinfecting agents. The change of biofilm bacteria from sensitive to resistant against disinfecting agents occurred between 2 and 3 weeks of biofilm maturation. The results emphasized the importance of knowing the maturation timeline of each biofilm model used to test the effectiveness of endodontic disinfecting agents against biofilm bacteria.

1.11.2 Biofilm growth in root sections and dentine disks

The use of root sections has been considered as a more clinically relevant substrate for biofilm growth. Clegg *et al.* (2006) used hemisections of root apices and samples from intracanal contents from patients diagnosed with chronic apical periodontitis were cultured to generate a polymicrobial biofilm. Each biofilm was immersed in 6% NaOCl, 3% NaOCl, 1% NaOCl, 2% CHX, 1% NaOCl followed by BioPure MTAD and sterile PBS and examined under SEM. The immersion of specimens showed that 6% NaOCl was the only irrigant capable of both rendering bacteria nonviable and physically removing the biofilm, followed by 1% NaOCl, 1% NaOCl combined with MTAD, whereas 2% CHX was not capable of disrupting the biofilm.

The use of dentine blocks to study endodontic disinfection against biofilms has been considered as a viable approach to simulate experimental conditions close to the *in vivo* reality of root canal areas, where irrigant remains stagnant and solution exchange is not effectively performed.

Liu *et al.* (2010) were the first to investigate the biofilm formation capability of *starved E. faecalis* cells on human dentine and the susceptibility of the biofilm to 5.25% NaOCl. *E. faecalis* American Type Culture Collection (ATCC) 29212 in different growth phases were incubated on human dentine and polystyrene blocks. SEM and biofilm assay were used to investigate the biofilm formation capability of these cells. The susceptibility of the biofilm to 5.25% NaOCl was further determined by the plate count method. SEM and biofilm assay showed that starved cells

were able to form biofilm on dentine with reduced efficiency as compared with the cells in the exponential phase and stationary phase. Biofilms of starved cells were more resistant to 5.25% NaOCl than those of stationary cells, and the impact of 5.25% NaOCl on them decreased as the biofilm matured.

Del Carpio-Perochena *et al.* (2015) investigated whether variation in pH of NaOCl increased its antibacterial and dissolution ability on polymicrobial biofilms formed *in situ*. The prepared dentine blocks were intraorally infected by using a removable orthodontic device for 48h and incubated in BHI for 48h to standardize the biofilm growth. The specimens were irrigated with 1 and 2.5% NaOCl with pH levels of 5, 7, and 12 for 20 min. The control group was irrigated with distilled water. The cell viability and the bacterial volume were measured at the pre- and post-irrigation procedures. Five random areas of each sample were further analyzed with CLSM. The antibacterial ability of the NaOCl was dependent on the concentration and acidification of the solution. All the experimental solutions were able to decrease the biomass, except for the 1% NaOCl-pH 5 group. The acidification of NaOCl improved its antibacterial ability, but the dissolution effect was decreased.

Wang *et al.* (2012) compared the antibacterial effects of different disinfecting solutions on young and old E. faecalis biofilms in dentinal tubules using a novel dentine infection model and CLSM. The bacteria were introduced into the dentinal tubules by centrifugation. After 1 day and 3 weeks of incubation, the infected dentine specimens were subjected to 1- and 3- min of exposure to 2% NaOCl, 6% NaOCl, 2% CHX and QMiX. Significantly fewer bacteria were killed in the 3-week-old dentin biofilm than in the 1-day-old biofilm. Three minutes of exposure resulted in more dead bacteria than 1 minute of exposure for both biofilms in all experimental groups. NaOCl 6% and QMiX were the most effective disinfecting solutions against the young biofilm, whereas against the 3-week-old biofilm, 6% NaOCl was the most effective followed by QMiX. NaOCl 2% was equally effective as 2% CHX. Within dentinal tubules, bacteria in established biofilms were less easily killed by endodontic disinfectants than bacteria in young biofilms.

Yang *et al.* (2016) evaluated the effect of the source of biofilm bacteria on their susceptibility in dentinal tubules to disinfecting solutions using an infected dentine model. Infected dentine blocks were prepared after *E. faecalis* strains VP3-181 and Gel 31 were introduced into dentinal

tubules by centrifugation to form mono-species biofilms. Three additional specimens of pooled plaque bacteria collected from different donors were used to grow multispecies biofilms in dentine. After 1 and 3 weeks of incubation, the samples were subjected to sterile water, 2% CHX, and 2% NaOCl for 3min and were assessed by viability staining and CLSM. The proportion of killed bacteria in mature (3 weeks) mono- and multispecies biofilms was lower than in young biofilms (1 week) after treatment. NaOCl 2% was more effective against multispecies biofilms in dentine than 2% CHX, whereas no significant difference was detected between 2% CHX and 2% NaOCl against the E. faecalis strains. Mature mono- and multi- species biofilms in dentinal tubules are more resistant to disinfectants than corresponding young biofilms. The susceptibility of the mono-species E. faecalis dentine biofilm showed strain-related differences, whereas the multispecies biofilms from different donors showed similar susceptibility.

1.11.3 Biofilm growth in root specimens and effectiveness of dynamic irrigation

The results of bioflm exposure to irrigants in a static manner should be critically appraised, as the crucial parameter of irrigant flow and the existing dynamics are omitted. Various experimental models have been proposed for the simulation of dynamic irrigation procedures in order to reproduce clinically relevant conditions. These include intact root specimens (Azim *et al.* 2016) or adjustable hemi-sected root halves (Bhuva *et al.* 2010, Lin *et al.* 2013, Niazi *et al.* 2014), 3-D printed transparent methacrylate (Mohmmed *et al.* 2016) or soft lithography-based models microfabricated from polyethylene glycol–modified polydimethylsiloxane root models (Layton *et al.* 2015) and transparent models from solidified polydimethylsiloxane (Macedo *et al.* 2014).

Quantitative measurement of viable counts following paper point sampling (Niazi *et al.* 2014), combined paper point sampling and qPCR (Alves *et al.* 2016), SEM (Bhuva *et al.* 2010, Lin *et al.* 2013, *Bao et al.* 2017), analysis with CLSM (Niazi *et al.* 2014, Wong & Cheung 2014, Azim *et al.* 2016), crystal violet assay (Layton *et al.* 2015), and real-time recording of biofilm fluorescence (Mohmmed *et al.* 2016) have been considered as acceptable methods to test antimicrobial killing or biofilm disruption efficacy, when a biofilm model is subjected to irrigation procedures. Macedo *et al.* (2014) were first to propose the use of a validated biofilm mimicking

gel, which had biomechanical properties similar to a microbial biofilm. However, each methodology has inherent limitations and the proper selection should be based on the scopes of the study to examine the antimicrobial capacity of irrigants on a microscopic or macroscopic level.

A two-phase study evaluated the disinfecting ability of chemomechanical preparation with rotary NiTi instruments and 2.5% NaOCl, combined with agitation with XPEF or UAI, in the root canals of extracted mandibular molars by means of a correlative analytical approach (Alves *et al.* 2016). In the first phase of the experiment, root canals were contaminated with *E. faecalis*, disinfected and sampled with paper points before and after chemomechanical preparation and after agitation with XPEF or UAI. Micro–computed tomography was used pre- and post-operatively to show whether the percentage of unprepared areas correlated to bacterial counts. The results of the first phase showed that the main root canal preparation resulted in substantial decrease of bacterial counts in both groups and the use of XPEF led to a further small bacterial, which was significant compared to UAI. Correlative analysis revealed no statistically significant relationship between bacterial reduction and the percentage of unprepared areas. In the second phase, the same teeth were re-contaminated and the same irrigation and agitation protocols were used. Samples from the isthmus area of mesial roots and the apical 5-mm were cryopulverised and DNA extraction was performed for quantification of *E. faecalis* cells by using a 16S rRNA gene-targeted qPCR assay. In phase 2, both methods had significant antibacterial effects in the main canal, but none of them could predictably disinfect the isthmus/recess areas.

Bhuva *et al.* (2010) compared the efficacy of conventional irrigation and UAI of 1% NaOCl against intraradicular *E. faecalis* biofilms in extracted standardized root halves which had been longitudinally sectioned and re-approximated during irrigation procedures. The root halves were processed for SEM and three images, coronal, middle and apical, were taken using a standardized protocol, randomized and biofilm coverage was assessed independently by three calibrated examiners, using a four-point scoring system. There were no significant differences in the scores for remaining biofilm coverage between conventional syringe irrigation with 1% NaOCl and UAI with 1% NaOCl at the three observed levels. However, the applied silicon index to retain root halves proximal was not tight and the authors highlighted the need to improve the testing apparatus.

In another study, a similar model which consisted of split root halves, adjustable in a steady custom block was created (Lin *et al.* 2013). In the root halves, a 0.2-mm-wide groove was placed in the apical 2 to 5 mm of the canal and a mixed bacteria biofilm was grown inside the canal under anaerobic conditions. Teeth were prepared with K-files, rotary NiTi files and the SAF and irrigation with 10ml 3% NaOCl and 4ml 17% EDTA. The examination of the areas inside and outside the groove was performed with SEM. No technique was able to remove all bacteria, but for all groups, significantly more bacteria were removed outside the groove than inside. The use of SAF significantly reduced more bacteria within the apical groove compared to hand and rotary instrumentation. In a study performed by the same research group, using the previous model, the effectiveness of the continuous or intermitted use of XPEF was compared to conventional needle irrigation and UAI (Bao *et al.* 2017). The two XPEF groups showed the best biofilm removal efficacy both inside and outside the groove followed by the 2 UAI groups. The authors concluded that the use of XPEF, as an irrigation agitation technique, may help to remove biofilm from hard-to-reach areas in the root canal system (Bao *et al.* 2017).

The use of CLSM for the localization and quantification of live and dead bacteria in human *ex vivo* mineralized dentinal tubules was introduced by Parmar *et al.* (2011). The use of fluorescent viability staining was claimed to be convenient, accurate and reproducible. However, the principal problems of this technique are related with the need to standardize fluorescence between optical slices to achieve consistent balance between the red and green signals. Consequently, the CLSM instrument parameters require individual adjustments for each image caption.

Niazi *et al.* (2014a, 2014b) used standardized longitudinally sectioned root halves which had been and re-approximated in a silicon index, similar to a previous study (Bhuva *et al.* 2010). In addition, two artificial lateral canals in middle and apical root third were created with the aid of rotary files, but their dimensions were not efficiently standardised. Nutrient-stressed multi-species biofilms were grown on the root halves, which were re-approximated and treated with proteolytic enzymes such as trypsin and proteinase K, combined with CHX, with or without UAI. The biofilms were investigated using CLSM with live/dead staining and quantitative microbial cultures. The results of both studies concluded that the application of UAI combined with proteolytic enzymes

and CHX was effective in reducing the biofilm substratum coverage. Quantitative microbial viable counts further disclosed the antimicrobial inefficiency of the irrigants in the lateral canals.

The extension of the bactericidal effect of 0.5% and 3% NaOCl into dentinal tubules, infected by a dual-species biofilm, including *E. faecalis* and *P. gingivalis*, was examined with the use of CLSM (Wong & Cheung 2014). The canal walls were irrigated with 3ml 0.5% or 3% NaOCl and the roots were split horizontally at 5-,7-, and 9-mm levels from root apex. Both concentrations of NaOCl significantly reduced the number of live bacteria in the most superficial layer (0.1mm) of root canal dentine, compared with the control (irrigation with sterile saline). Irrigation with 3% NaOCl resulted in significantly lower amounts of viable bacteria than 0.5% NaOCl, for the next 2 layers (100–300μm into dentinal tubules). However, in deeper tubule lengths (>300μm), bacteria could not be completely eliminated (Wong & Cheung 2014).

A novel CLSM analysis was proposed by Azim *et al.* (2016) to determine the efficiency of conventional irrigation, sonic agitation, XPEF and PIPS in eliminating bacteria from dentinal tubules. The collected human root specimens were prepared and inoculated with *E. faecalis* for 3 weeks. Canals were then disinfected with 2ml 17% EDTA for 1min and 3ml 6% NaOCl with the aforementioned agitation protocols. The bacterial reduction in the canal was determined by MTT assays, whereas for measuring live versus dead bacteria in the dentinal tubules, teeth were split open and the coronal, middle, and apical thirds of the canal dentine were scanned by CLSM, to determine the ratio of dead/total bacteria in the dentinal tubules at various depths. All 4 irrigation protocols significantly eliminated bacteria in the canal and XPEF had the greatest bacterial reduction. CLSM analysis showed that XPEF had the highest level of dead bacteria in the coronal, middle and apical segments at 50-mm depth. PIPS was the most effective in killing bacteria at the 150-mm depth.

Thorough understanding of fluid dynamics in root canal irrigation and corresponding antibiofilm capacity may support improved disinfection strategies. The study conducted by Layton *et al.* (2015) aimed to develop a standardized, simulated root canal model that allows real-time analysis of fluid/irrigation dynamics and its correlation with biofilm elimination. The authors used a maxillary incisor with an instrumented root canal, imaged with micro–computed tomography. The canal volume was reconstructed in 3-D and replicated in PEG-modified PDMS models.

Irrigation protocols included syringe conventional irrigation and intermittent or continuous UAI (IUAI, CUAI). Real-time fluid movement within the apical 3mm of canals was imaged by using microparticle image velocimetry. Syringe irrigation generated high velocity and shear stress in the apical 1-2mm. IUAI generated consistently low shear stress in the apical 3mm. CUAI generated consistently high levels of velocity and shear stress in the apical 0-1 and 2-3mm. In the same models, the canals were inoculated with *E. faecalis* to grow 3-week-old biofilms. Biofilm reduction by irrigation with SI, CUAI and IUAI was assessed by using a crystal violet assay and compared with an untreated control. Biofilm was significantly reduced only by CUAI compared with the control. Overall, the use of CUAI exhibited the highest mechanical effects of fluid flow in the apical 3 mm, which correlated with significant biofilm reduction. The proposed novel model good apply for a valid method for study of correlations between fluid dynamics and the antibiofilm capacity of root canal irrigation methods.

The fabrication of a novel 3-D printed transparent methacrylate tooth model of standardised dimensions (18mm length, apical size 30, a .06 taper and a lateral canal of 3mm length, with 0.3 mm diameter) was recently proposed as an alternative to the use of human teeth and the relevant root canal variability and availability issues (Mohmmed *et al.* 2016). The physico-chemical properties such as zeta-potential, wettability and surface free energy of stereolithography materials were compared to dentine, as well as the potential of each material to develop *E. faecalis* biofilm on their respective surfaces (Mohhmed *et al.* 2017). The tested materials showed similar physico-chemical properties to dentine. A series of studies was performed to investigate in situ the biofilm removal from the apical third and lateral canal of a simulated root canal system. After 60s irrigation using syringe and needle of 9ml 2.5% NaOCl, the irrigant was either left stagnant in the canal or activated using gutta-percha, sonic or ultrasonic methods for 30s. The models were observed under a fluorescence microscope and images were then captured every second using an external camera. Biofilms were stained using crystal violet for visualization. The residual biofilm percentages were measured using image analysis software. All agitation groups reduced the available chlorine and pH of NaOCl more than that in the syringe irrigation group (Mohhmed *et al.* 2016). Ultrasonic agitation of NaOCl left the least amount of residual biofilm in comparison to sonic and gutta-percha agitation methods in the main as well in the lateral canal (Mohhmed et al. 2016, Mohhmed et al. 2018). Residual biofilm was less using 5.25% than with

2.5% NaOCl and biofilm removal was evident with the needle placed 2mm to the canal terminus (Mohhmed *et al.* 2017). Finally, following irrigation, the observation of residual biofilms using CLSM, SEM and TEM indicated that total biofilm degradation and nonviable cells were associated with the UAI group (Mohhmed *et al.* 2017b).

In 2014, Macedo *et al.* introduced and characterized a reproducible hydrogel as a suitable biofilm mimic in endodontic research. A rheometer was used to characterize the viscoelastic properties and cohesive strength of the hydrogel for suitability as a biofilm mimic and confirmed that the hydrogel demonstrated viscoelastic behaviour with mechanical properties comparable to real biofilms. The authors produced transparent root canal models by solidifying polydimethylsiloxane around a finger spreader, as well as an isthmus (width 3mm, thickness 0.15mm, length 4mm) at 2–5mm from the spreader tip, or a lateral canal (diameter 0.25mm, length 5mm) at 3mm from the spreader tip. The main root canal in these models had a length of 18mm, apical diameter of 0.35 mm and a taper of 6%. The hydrogel was injected into the isthmus or lateral canal using a 30G needle and left to solidify for at least 1min at room temperature, whilst the root canal models were positioned in front of a microscope. The removal of the hydrogel was attempted with syringe irrigation or UAI and 8.7% NaOCl or distilled water. A high-speed camera was attached to a microscope and the recorded images were analysed with a digital software. The results of this study showed that UAI enhanced the cleaning effect of NaOCl in isthmi and lateral canals and a greater depth of cleaning was achieved within an isthmus than a lateral canal with UAI. On the contrary, NaOCl without UAI resulted in a greater depth of hydrogel removal from a lateral canal than an isthmus, due to the formation and entrapment of bubbles.

The application of validated transparent root models appears to be promising for the study of several irrigation parameters, dynamic phenomena and their relevant efficacy against grown biofilms or relevant biofilm mimics. However, the constitution of the models cannot compensate for the anatomy of root dentine and the presence of dentinal tubules, which create difficult-to-reach niches for residual bacteria to survive. Biofilm residues, attached on the root canal lumen and artificially prepared isthmuses and lateral canals were efffectively visualised, however their cell viability was not justified.

1.12 Inactivation of root canal irrigants

Many root canal disinfectants including NaOCl present excellent antimicrobial activity *in vitro* whereas *in vivo* they often fail to completely kill all microbes. This may be related to organic and inorganic matter present in the canal, which can interact with NaOCl thereby reducing efficacy, however little information is available. A study by Haapasalo *et al.* (2000) showed that dentine powder had an inhibitory effect on the antimicrobial activity of 1% NaOCl against *E. faecalis*, which was dependent on the concentration of the disinfectant as well as on time of incubation of the dentine powder before addition of bacteria (Haapasalo *et al.* 2000).

Albumin is a main protein in human serum and is present in inflammatory exudates, gingival crevicular fluid and dentinal fluids (Bickel *et al.* 1985, Mäkelä *et al.* 1991, Knutsson *et al.* 1994). Sassone *et al.* (2003) reported the absence of inhibitory effect of 0.5% BSA against the antimicrobial activity of 1% and 5% NaOCl. However, in another study, a higher BSA concentration (6.7%), presented an inhibitory effect on NaOCl (0.08-6%) against all tested planktonic microbial species (*E. faecalis, E. Coli, C. albicans, Staph. Epidermis*) (Pappen *et al.* 2010). The inhibition was dependent on the relative concentrations of both NaOCl and BSA (Pappen *et al.* 2010).

In summary, factors that continue to pose challenges in the irrigation and disinfection of root canals include, biofilm resistance (Mah *&* O'Toole 2001, Svensäter *&* Bergenholtz 2004), poor penetration of irrigant (Shen *et al.* 2009), low concentration (Siqueira *et al.* 1999), short exposure time (Shen *et al.* 2009, Williamson *et al.* 2009), small overall delivery volume (Moorer & Wesselink 1982), and poor exchange of irrigants in the highly complex root canal anatomy (isthmus areas, dentinal tubules and accessory canals) (Dammaschke *et al.* 2004, Gao *et al.* 2009).

1.13 Interaction of NaOCl with pulp tissue

The interaction of NaOCl with pulp tissue has been widely explored, given its use as main root canal irrigant. Several laboratory studies have focused in the investigation of clinical parameters affecting tissue-dissolving ability of NaOCl. A heterogeneity in the protocols set-up

and the applied methodology for the assessment of dissolving capacity was evident. In the majority of the studies, there was a direct contact of the soft tissue specimens with the irrigant solution.

Tissues from a number of different sources have been used in studies about NaOCl tissue-dissolving ability (Clarkson *et al.* 2006). Porcine muscle tissue (Hasselgren *et al.* 1988, Christensen *et al.* 2008), rabbit liver (Moorer & Wesselink 1982), rat connective tissue (Hand *et al.* 1978), pig palatal mucosa (Naenni *et al.* 2004), bovine muscle tissue (Turkun & Cengiz 1997) and bovine pulp (Okino *et al.* 2004) have been used to determine dissolution ability of different irrigants. Fresh bovine pulp tissue is a close simulative approach to represent the available organic remnants in root canals because of its similarity to human pulp tissue (Clarkson *et al.* 2006). The use of different types of tissues instead of dental pulp was associated with their prominent availability and the easier standardization of the specimens' surface area (Turkun & Cengiz 1997).

The results of the studies have been expressed as percentages of mean weight loss of soft tissue specimens at a certain time points, or during different observation periods, or as the minimum time required for the complete disintegration of soft tissue specimens. As an alternative approach, the amount of dissolution was expressed according to the levels of detectable proteins such as hydroxyproline, which is profound in collagen, synthetic hydroxyapatite or human serum albumin (Koskinen *et al.* 1980).

The results of such laboratory studies are not always accurately comparable and cannot be extrapolated in the dynamic conditions that exist in complex root canals, during instrumentation and irrigation (Moorer & Wesselink 1982, Beltz *et al.* 2003). The pioneer study of Senia *et al.* (1971) was the first to document the anatomical constrictions of the root canal system may compromise the tissue-solvent action of NaOCl. The two mesial root canals of human mandibular molars were used to evaluate the remaining pulp tissue content when NaOCl 5.25% v/v or distilled water were used. Cross sections of the roots were made at 1 mm, 3 mm and 5 mm from the apex and, after staining with basic fuchsin, were examined microscopically at x100. Any pulp tissue present was stained red. The results of the study showed that limited surface contact, volume and exchange of irrigant may limit the effectiveness of NaOCl at the 1mm and 3mm levels. The surfaces of remaining pulp tissue could not be exposed simultaneously to the solvent action of

NaOCl. Without direct contact, the deeper protected tissues were dissolved slowly and less completely. The pulp tissue remaining after instrumentation was attached to the canal walls or sheltered within an isthmus. NaOCl was is more effective at the 5mm level, where the canal was wider and a greater volume and exchange of solution are possible.

Neelakantan *et al.* (2016) aimed to evaluate the efficacy of a new irrigation system (EndoIrrigator Plus; Innovations Endo, Nasik, India) using a histologic method *ex vivo*. Mandibular molars with a normal pulp extracted for periodontal reasons were assessed for the presence of an isthmus using cone-beam computed tomographic imaging. The root canals were initially instrumented up to a ProTaper F2 instrument using 3% NaOCl in a closed apical design. Final irrigation delivery/activation was performed with syringe (positive pressure), apical negative pressure delivery with continuous warm activated irrigation and evacuation system (CWAIS), manual dynamic agitation and UAI. The isthmus regions (1, 3, and 5mm from the apex) were analyzed by hematoxylin-eosin stain to calculate the percentage of remaining pulp tissue relative to the area of the isthmus. None of the methods could completely clean the isthmus. CWAIS left behind the least amount of RPT compared to the other groups at all 3 root levels, whilst manual dynamic agitation showed significantly less remaining pulp tissue at 1 and 3mm from the apex compared with UAI and syringe irrigation. However, no significant difference was observed between UAI and manual dynamic agitation at the 5-mm level.

The significant contribution of agitation with ultrasonics has been highlighted in a recent *in vitro* study, which compared the effectiveness of 5.25% NaOCl with three activated irrigation techniques to remove pulp tissue from the isthmus of a transparent tooth model (Malentacca *et al.* 2018). The three techniques assessed were: the EndoVac, UAI and ultrasonic wave aspiration (TUWA). Conventional syringe irrigation was used as a control. The applied transparent tooth model used the mesial root of an extracted mandibular first molar that had an isthmus and two independent mesial canals. After preparation, the root canals were filled with fuchsine-stained bovine pulp tissue. Photographs were taken and analysed using an imaging software. The application of UAI and TUWA were the most effective techniques at removing artificial pulp tissue from the isthmus of a transparent tooth model. The main limitation of the current model was that the bovine pulp tissue was packed into the root canal system and therefore lacked any physical attachment to the root canal dentine, which occurs with the biofilm phenotype.

A stained collagen *ex vivo* tooth model was introduced by Huang *et al.* (2007) to evaluate the effect of several parameters -including location in the root canal, root canal preparation (size) and irrigation parameters (volume, agitation)- on dissolution capacity of 2.5% NaOCl. Human teeth with single straight canals were prepared to different apical sizes (20, 40) and tapers (0.04, 0.08) and split longitudinally into two. Stained collagen was applied to the canal surfaces and the specimens were reassembled in a silicone matrix for static or dynamic (manual pumping) irrigation. Digital images of the canal surface were taken before and after irrigation with 9, 18, 27 and 36ml solution. The percentage of canal surface covered with stained collagen was quantified with a digital software. The results of this study showed that all variables had a significant influence on outcome of irrigation and collagen removal. The corono-apical level of canal was the most dominating factor; after irrigation, the apical third had 19.9% and 33.8% less area covered with collagen than the middle and coronal thirds respectively. The stained collagen film could not be removed completely by either static or dynamic irrigation. Factors influencing removal, in rank order of decreasing priority, were: corono-apical level, apical size and taper of canal preparation and dynamic/static irrigation.

Bryce *et al.* (2018) used the same stained collagen tooth model *ex vivo*, to evaluated the efficacy of sonic irrigation (EndoActivator®) using various polymer tips and power settings. The root canals of fifty human, straight single-rooted extracted teeth were initially prepared and the roots were split longitudinally, stained collagen applied to the canal surfaces, photographed and re-assembled. The canals were subjected to syringe irrigation with or without supplementary sonic agitation, using different tips and power settings. They concluded that supplementary sonic irrigation resulted in significantly less residual collagen compared with syringe irrigation only.

Estevez *et al.* (2017) evaluated the porcine palatal mucosa dissolution from artificial grooves using a final rinse with NaOCl with or without a surfactant or UAI. The root canals of human maxillary central incisors were chemomechanically prepared and the teeth split. A standardized longitudinal intracanal groove was created in 1 of the root halves. Porcine palatal mucosa samples were collected, adapted in order to fit into the grooves, and pre-weighed. The reassembled specimens were randomly divided in 3 experimental groups based on their agitation protocol (15s or 30s UAI) and type of NaOCl preparation, including a 6% solution with or without surfactant). An EDTA intermediate rinse was included. Palatal mucosa weights were re-

measured after the assays. The results showed that complete dissolution did not occur in any sample. The addition of a surfactant to NaOCl and/or UAI increased palatal mucosa dissolution within artificial grooves in the root canal of incisor teeth. UAI was often able to compensate for the absence of surfactants.

Conde *et al.* (2017) used the previously described tooth model to compare the soft-tissue dissolution by NaOCl, with an EDTA intermediate rinse, with or without activation with UAI or sonic activation using the Endoactivator or Eddy tips. The solutions were delivered using a syringe and needle 2 mm from working length. Total irrigation time was 150s, including 60s of activation. Weight loss occurred in all experimental groups. Irrigant activation resulted in greater weight loss when compared to the nonactivated group, with no significant differences amongst the different activation systems. The study confirmed that agitation increased the tissue-dissolving activity of irrigants from artificial grooves in root canals of maxillary central incisors.

A recent study was designed to evaluate the effectiveness of NaOCl, NaOCl-EDTA and NaOCl + HEBP activated by ultrasonics and XPEF on organic tissue removal from simulated internal root resorption cavities, in single rooted teeth. The root canals of the specimens were instrumented, split longitudinally and semi-circular cavities were prepared in the canal walls on each half of the roots. Samples obtained from ground bovine muscle tissue were weighed and adapted into the semi-circular cavities. The root fragments were reassembled and cemented to create a circular simulated resorption cavity within the canal. When irrigation and agitation protocols were completed, the teeth were disassembled and the tissue remaining inside the resorption cavities were re-weighed. The use of XPEF with the experimental solutions resulted in the greatest tissue weight loss. The use of a NaOCl + HEBP mixture activated with XPEF was an effective irrigation regimen for removing simulated organic tissues from artificial internal root resorption cavities.

A contributory parameter to the limited dissolving efficacy of NaOCl in clinical conditions is related to its inactivation when it interacts with organic matter. Moorer & Wesselink (1982) evaluated the reactivity of sodium hypochlorite in homogeneous and non-homogeneous solutions containing organic matter. The varying concentrations of NaOCl (0.6-3.00 % w/v) lost their available chlorine content after mixing in hydrolysate solution (homogeneous solution) or rat liver fragments (non-homogeneous solution). When an excess of organic matter existed, the activity

of NaOCl was rapidly depleted and the pH was lowered. The contribution of agitation with ultrasonics resulted in rapid dissolution of tissue specimens as well as inactivation of chlorine content (Moorer & Wesselink 1982). Considering the amount of organic matter in the root canal system and the small volume of NaOCl that can be applicable, the best way to optimize its effectiveness is the frequent replenishment with new fresh solution, the agitation of the solution and or the use of high concentrations.

A similar study was performed for the evaluation of bovine pulp tissue dissolution capacity and its correlation with residual active chlorine content in a dynamic environment of continuous irrigant flow (Spano *et al.* 2001). Residual chlorine of four NaOCl solutions (0.5%, 1%, 2.5%, 5% w/v) was measured at the time when pulp fragments were completely disintegrated. The results of the study showed that the speed of dissolution was proportional to the initial concentration. In addition, chlorine consumption and pH reduction were more intense in lowest concentrations (Spano *et al.* 2001).

A review of literature suggests that NaOCl have a superior dissolution capacity compared to CHX and EDTA (Naenni *et al.* 2004, Okino *et al.* 2004, Clarkson *et al.* 2006, Stojicic *et al.* 2010). A general consensus is that concentration, volume, pH, temperature, time, tissue-irrigant contact area/surface, mechanical action-agitation, frequency of irrigant replenishment, amount of organic matter in relation to amount of irrigant in the system and canal preparation size have a significant influence on the dissolution capability of NaOCl (Moorer & Wesselink 1982, Clarkson *et al.* 2006, Stojicic *et al.* 2010).

1.14 Interaction of root canal irrigants with dentine

Dentine is a substrate with a complex inorganic and organic structure and as a mineralized connective tissue has composition and mode of formation similar to that of bone. Dentine structure is a complex hydrated composite, consisting of mineralized inter-tubular dentine, highly mineralized peritubular dentine and microtubules filled with dentinal fluid and odontoblastic processes (Pashley 1989, Marshall 1993).

The major inorganic components of dental hard tissue are calcium (Ca) and phosphate (P) present in hydroxyapatite crystals. The inorganic phase of dentine represents 70% of its content and is composed of carbonated apatite located in the intra- and inter- fibrillar spaces of collagen matrix, protecting mineralised dentine from thermal denaturation and enzymatic degradation (Armstrong *et al.* 2006, Carrilho *et al.* 2009).

The organic phase of dentine represents 30% of its content and is organised in a hydrated organic matrix, most of which consists of type I collagen that provides viscoelasticity, toughness and fatigue resistance (Lucchese *et al.* 2008). In addition to type I collagen, which contributes up to 90% of the organic material, the extracellular matrix (ECM) of dentine contains several proteins and proteoglycans (PGs), collectively referred to as non-collagenous proteins (NCPs) constituting approximately 10% of the matrix (Buttler & Ritchie 1995).

The role of NCPs has been considered pivotal for the processes involved in dentinogenesis (Linde 1989) as consistent evidence indicates that NCPs co-ordinate in mineral deposition within the extracellular dentin matrix, as well as actively promote and control collagen fibril mineralization and crystal growth within pre-dentine, when this tissue is converted into dentine (Qin *et al.* 2004, Qin *et al.* 2007, Tjäderhane & Haapasalo 2012).

During root canal irrigation, the use of endodontic disinfectants such as NaOCl, EDTA and CHX result in chemical interactions with radicular and coronal dentine, whilst the irrigants flow in the root canal system or remains deposited in the pulp chamber.

1.14.1 Interaction of NaOCl with dentine

The deproteinizing effect of NaOCl in the organic dentine structure has been clearly documented in several studies. The results of imaging studies including SEM, TEM, PLM microscopy (Moreira *et al.* 2009, Zhang *et al.* 2010b), x-ray diffraction analysis (Ozdemir *et al.* 2012), immunohistochemistry (Oyarzun *et al.* 2002) and FTIR spectroscopy (Di Renzo *et al.* 2001, Hu *et al.* 2010, Zhang *et al.* 2010a) have shown that NaOCl interacts with the organic phase of dentine leading to concentration-dependent and time-dependent collagen depletion.

As a non-specific oxidizing and proteolytic agent, NaOCl oxidizes the organic matrix and denatures the collagen components of the smear layer. The use of attenuated total reflectance Fourier transform-infrared spectroscopy (ATR-FTIR) or FTIR spectroscopy is commonly employed to analyse the amide:phosphate ratio changes in dentine, following the interaction with NaOCl (Hu *et al.* 2010, Zhang *et al.* 2010a, Zhang *et al.* 2010b). The results of the short-term (1-, 5-, 10- min) effect of 1ml NaOCl at different concentrations (0.5%, 1%, 2.25%) against dentinal slabs showed that the amide:phosphate ratio decreased significantly as compared with the control group (0.9% NaCl) (Hu *et al.* 2010). Moreover, the assessment of the long-term (10-240min) NaOCl interaction with the organic phase of dentine powder resulted in concentration-dependent and time-dependent collagen depletion (Zhang *et al.* 2010a, Zhang *et al.* 2010b).

The use of SEM showed that the superficial destructive effect of NaOCl on mineralized dentin of instrumented root canals was irreversible and occured regardless of the use of EDTA as final irrigant (Zhang *et al.* 2010b). This indicates that low and high NaOCl concentrations are capable of removing the organic phase from the “superficial subsurface” of mineralized dentine, creating an apatite-rich, collagen-sparse dentine subsurface (Driscol *et al.* 2002, Mountouris *et al.* 2004). The depth of the affected dentine has been justified with use of TEM, following the instrumentation and irrigation of a root canal with 1.3% or 5% NaOCl and a final rinse with 17% EDTA. The use of 1.3% NaOCl and 17% EDTA did not cause dentinal erosion on the surface of instrumented canal walls. The use of TEM further showed dissolution of the smear layer and the presence of an intact intertubular dentine surface with exposed tubular orifices (Zhang *et al.* 2010). After the intermittent use of 5% NaOCl for 20min, subsurface erosion was present and extended approximately 10–15μm into the underlying dentine with widening and connection of the intertubular spaces (Zhang *et al.* 2010). The presence of this collagen-sparse subsurface zone may also create non-uniform deproteinization channels (Di Renzo *et al.* 2001), which could facilitate subsequent EDTA penetration and further apatite dissolution. However, the depth of demineralization of EDTA did not increase and was limited to 0.5μm or less in all the eroded surface and subsurface regions. Overall, eroded dentine is considered to be more brittle than untreated mineralized dentine (Marendig *et al.* 2007).

The application of NaOCl with different concentrations and exposure time does not affect the inorganic components of dentine (Hu *et al.* 2010, Ozdemir *et al.* 2012). NaOCl removes the

organic components but does not influence the inorganic phase of human dentine. The results of these studies are in agreement with qualitative findings of others studies (Di Renzo *et al.* 2001, Driscoll *et al.* 2002, Mountouris *et al.* 2004).

One of the side effects of the interaction of NaOCl with dentine that has received relatively little attention in the endodontic literature is the impact on its biomechanical properties and matrix (Oyarzun et al. 2002). A comprehensive review alluded to adverse biomechanical effects on properties of root dentine when used as an endodontic irrigant (Pascon *et al.* 2009). A 60-sec exposure to 5% NaOCl caused the reduction in dentin microhardness (Saleh & Ettman 1999). A 15-min exposure to 1% NaOCl reduces the dentine microhardness (Oliveira *et al.* 2007). A 24-min exposure to 2.5% NaOCl causes a significant drop in the flexural strength (Marendig et al. 2007). The 2-h exposure on 5.25% NaOCl reduces the flexural strength and elastic modulus of dentine (Sim *et al.* 2001). The decline in flexural strength is probably attributed to the generation of a brittle layer of apatite crystallites that are not supported by a structurally intact collagen matrix. It is well-known that the mineral component in hard connective tissues contributes to strength and elastic modulus, whereas the collagen component is responsible for toughness of the tissues (Wang *et al.* 2001). Destruction of the collagen matrix in mineralized tissues results in a less tough, more brittle substrate that might precipitate fatigue crack propagation during cyclic stresses (Kruzic & Ritchie 2008). This might increase the susceptibility of the root-treated teeth to post-treatment crown or root fracture.

1.14.2 Reactivity loss of NaOCl in contact with dentine

The chemical efficacy of NaOCl solutions in contact with dentinal axial walls has gained little focus in international literature. Equally with NaOCl interaction with soft tissues, organic matter and pulp tissue, NaOCl interacts with dentine resulting in a loss of total available chlorine. Macedo *et al.* (2010) measured the average velocity of chlorine consumption and defined it as reaction rate (RR), which can be determined by the quotient between the difference in the concentration of NaOCl before and after exposure time (D[NaOCl]) and the total exposure time (Dt) (RR = D[NaOCl]/Dt).

In a bovine incisor model, The RR of the NaOCl solutions was assessed by measuring the amount of total available chlorine (%v/v) in solution before and after exposure to bovine dentine, using a standard iodine/thiosulfate titration method (Macedo *et al.* 2010). The RR of different concentrations and pH values of NaOCl were assessed after exposure to bovine dentine and application of different irrigant activation protocols, including UAI and laser activated irrigation (LAI). The results of the study showed that an association exists between the RR of NaOCl solutions and the activation, activation type and concentration of NaOCl (Macedo et al. 2010). The concentration of NaOCl solutions did not affect the percentage of chlorine loss but only the amount of chlorine consumed. Activated NaOCl solutions (UAI and LAI) showed a higher RR than non-activated NaOCl solutions. The results of a recent study, using the same protocol, showed that the RR of NaOCl with dentine can be further enhanced by refreshment as well as exposure time (Macedo *et al.* 2014).

1.14.3 Interaction of EDTA with dentine

Apart from the removal of the inorganic compounds of smear layer, the application of EDTA demineralizes only the inorganic components of surface dentine via calcium chelation (Oyarzun *et al.* 2002, Di Renzo *et al.* 2001, Saito *et al.* 2008). It has been well documented that dentine collagen degradation from mineralized dentine is not associated with the demineralization caused by the use of EDTA, when it is used as the final active irrigant (Zhang *et al.* 2010).

EDTA removes the collagen-depleted apatite phase to expose the underlying cause of destruction that is morphologically perceived as canal wall erosion. Erosion of crown dentine that is conditioned with EDTA for bonding purpose has never been reported in the adhesive dentistry literature. Even for longer contact times (1–2min), that are clinically relevant for root canal irrigation, the calcium chelation capacity of EDTA is self-limiting because the chelating ions have reacted with dentine calcium ions, up to when an equilibrium is reached (Verdelis *et al.* 1999, Machado-Silveiro *et al.* 2004). Demineralisation is also arrested due to the excellent buffering capacity of dentine (Camps & Pashley 2000). Thus, it is unlikely that canal wall erosion is caused solely by EDTA irrigation.

This explains the findings of other studies in which the intermittent use of chelating agents and NaOCl solutions led to intermittent erosions of the canal walls, characterized by surface dissolution of intertubular and peritubular dentine (Torabinejad *et al.* 2003). As a conclusion, the erosion is derived from the use of NaOCl as the initial irrigant, with the detrimental effect related to high NaOCl concentrations and extensive contact times with intact radicular dentine.

A recent study showed that flexural strength of dentine did not decline after treatment with 17% EDTA only and presented similar values to control group (water immersion) (Zhang *et al.* 2010b). Considering all previous findings, the final use of 17% EDTA for a limited time to remove the smear layer does not appear to be contributory to the well-reported decline in mechanical properties of dentine, associated with the extensive use of NaOCl as a root canal irrigant (Pascon *et al.* 2014).

1.14.4 Interaction of CHX with dentine

The cationic nature of the CHX molecule is essential for its absorption by anionic substrates such as proteins and hydroxyapatite. This reaction is reversible and end in the release of CHX, leading to substantive antimicrobial activity and is referred to as “substantivity” (Khademi *et al.* 2008). This effect depends on the concentration of CHX. At concentrations exceeding 0.02%, a multilayer of CHX is formed on the surface, providing a reservoir which can rapidly release excess CHX into the environment as the concentration of the CHX in the surrounding environment decreases (Emilson *et al.* 1973, Souza *et al.* 2018).

The use of CHX as a final root canal irrigant has been recommended to enhance disinfection regime and promote substantivity. Currently, there are no available studies reporting any chemical effects of CHX against dentine. A recent ultramorphological ex vivo study showed that final irrigation with 2% CHX did not affect dentine structure, when examined with SEM and TEM, compared to modifications in dentine organic ultrastructure, characterized by the thinning of dentin collagen fibrils and erosion of the peritubular dentine, caused by NaOCl, enhanced by EDTA and/or UAI (Wagner *et al.* 2017).

In another study, time-of-flight secondary ion mass spectrometry (TOFSI-MS) was used to qualitatively evaluate the chemical characteristics of dentine surface and compare it with dentine blocks exposed to NaOCl, EDTA or CHX. The results confirmed that NaOCl removed protein components from the dentine matrix, EDTA removed calcium and magnesium ions, and CHX formed an adsorbed layer on the dentine surface with no residual chemical effects (Kolosowski *et al.* 2015).

The irrigation of extracted root specimens with CHX did not result in reduction of dentine microhardness and did not induce surface roughness modifications, compared to NaOCl or EDTA (Ari *et al.* 2004, Aslantas *et al.* 2014).

1.15 Interaction of NaOCl with periapical tissues

The risk of extrusion of debris, irrigant and microbes through the apical foramen into the periradicular tissues even under conditions of good clinical practice is a reality. NaOCl has gained significant attention due to its relatively high toxicity in contact with periapical tissues, even in a low-scale exposure in terms of time, quantity and concentration. A 5% NaOCl solution as well as several dilutions from ½ to 1/1000 was proved to be extremely caustic in contact with vital tissue ex vivo, causing hemolysis, eye irritation and skin ulceration following intra-dermal injections (Pashley et al. 1985). The direct contact of 5.25% NaOCl with dog femur bony specimens ex vivo caused degradation of the organic matrix, significant changes in cancellous structure, leaving large craters of apparent demineralisation and paucity of cancellous bone trabeculation and bone marrow (Kerbl *et al.* 2012). However, apart from NaOCl, any source of chemical, physical, mechanical, microbial irritation has a potential to disrupt the integrity and balance of periradicular tissues, leading to the induction of periapical inflammatory response (Siqueira 2003).

The histological examination of periapical tissues following irrigation with NaOCl disclosed the presence of immunoglobulins and mast cells (Naidorf 1985, Torabinejad *et al.* 1985), a mixture of necrotic pulp tissue, bacteria, bacterial fragments, irrigants, infected or non-infected dentine debris and tissue fluids, which is also representative of the smear layer formed during instrumentation (Torabinejad *et al.* 1985). Thus, the presence of chemically altered pulp tissue

proteins or root canal contents may result in the formation of antigen-antibody complex and release vasoactive amines respectively, which initiate an inflammatory response or aggravate an existing inflammatory process (Naidorf 1985, Torabinejad *et al.* 1985). In addition, other studies showed that the presence of either infected or non-infected dentine chips (Seltzer *et al.* 1968) as well as the presence of sterile connective tissue in the periapical tissues (Torneck 1967) may predispose for the immunological promotion of inflammatory reactions. Thus, even a small portion of extruded material may have a potentially high virulent or antigen capacity to induce inflammation. However, apart from the quantity and quality of the extruded content, it is still unclear whether or how the level of the host's resistance is associated and affected in cases of apical extrusion.

Several laboratory studies have been performed in an attempt to quantitate the amount of extruded debris during root canal chemomechanical preparation. The studied quantitative parameters were the extruded volume of liquid or irrigation solutions, the calculated mass of extruded dentine shavings and the population of extruded bacteria into artificial periradicular space (Beeson *et al.* 1998, Hauser *et al.* 2007, George & Walsh 2008, Tasdemir *et al.* 2008, Desai & Himel 2009, Mitchell *et al.* 2011). Many of the published studies present inherent methodological problems with regard to the simulation and validation of the periapical environment, as well as the sensitivity of the measurements and experimental procedures. The fundamental limitations were associated with atmospheric pressure equalization between the periradicular space and external air, with no exact scientific justification. As a result, the simulation of periapical tissues pressure and the inherent resistance to the occurrence of extrusion were omitted. The possibility of a tooth apex surrounded by ambient air *in vivo* is extremely unlikely, therefore an overestimation of debris and irrigant extrusion is highly expected, compared with in vivo conditions. The use of foams and gels was also adopted but introduced difficulties in the quantification of the extruded irrigant and qualitative or semi-quantitative evaluation was employed.

This limitation had a potential to overcome with the use of 1.5% sterile agarose, which was an acceptable medium in terms of simulating periapical tissue resistance and multi-purpose analysis with the aid of micro-CT and microbiological analysis with DNA extraction, to compare the levels of apically extruded bacterial and hard tissue debris (Alves *et al.* 2018). The distobuccal

canals from extracted maxillary molars were contaminated with E*. faecalis* and mounted in an apparatus that simulates the apical resistance offered by the periapical tissues and permitted to collect debris extruded during preparation. Saline was used as the irrigant during preparation and the XP-endo Shaper or Reciproc were used for instrumentation. DNA extracts from samples taken from the canal before and after preparation were subjected to quantitative real-time polymerase chain reaction for *E. faecalis* counting. The volume of extruded debris was evaluated by micro-computed tomographic imaging. DNA was further extracted from the extruded hard tissue debris and analyzed by quantitative real-time polymerase chain reaction. Both instruments produced a similar volume of extruded debris, but extruded bacteria counts were significantly lower with Reciproc than the XP-endo Shaper. However, no correlation was observed between the extruded bacterial counts and debris volume.

Recently, a series of comprehensive studies introduced a point conductivity probe, to measure the real-time periradicular extrusion of NaOCl, by calculating the available chlorine ion content in different simulations of periaradicular space *ex vivo* (Psimma *et al.* 2013a, *Psimma et al.* 2013b, Boutsioukis *et al.* 2013). The authors validated 4 different experimental conditions to simulate periapical tissue resistance including air-closed, air-open, water-closed, water-open environments (Psimma *et al.* 2013a). The results of the study showed that the water-closed and water-open periradicular environments may correspond in simulating high- and low- compliance periapical lesions. The absence of tissue resistance (air-closed, air-open) led to overestimation of apically extruded irrigant (Psimma *et al.* 2013a). In addition, the effect of several operative and anatomical parameters including the effect of needle type, insertion depth, irrigant flow rate, agitation technique, apical preparation size, apical constriction diameter, root canal curvature were investigated (Psimma *et al.* 2013a, Psimma *et al.* 2013b, Boutsioukis *et al.* 2013).

Open-ended needles resulted in more extrusion in comparison to close-ended needles. Extrusion decreased as needles moved away from root canal working length. A flow rate increase resulted in increased irrigant extrusion. Manual dynamic agitation extruded significantly more irrigant than sonic and ultrasonic agitation. Apical size preparation increase resulted in less extrusion, whereas the effect of apical constriction diameter and root canal curvature in irrigant extrusion were insignificant (Psimma *et al.* 2013a, Psimma *et al.* 2013b, Boutsioukis *et al.* 2013).

The proposed system appeared to be a promising innovation to overcome the methodological limitations of previously conducted studies testing apical extrusion.

An overview from the existing laboratory studies suggests that all instrumentation and irrigation techniques result in debris, irrigant and microbial extrusion regardless of the methodology (Tanalp & Güngör 2013). The use of hand files with a push-pull (filing motion) leads to significant debris extrusion in comparison to rotational movement (Al-Omari & Dummer 1995). In general, instrumentation techniques applied in a crown-down or step-down manner lead to significant less debris extrusion (Fairbourn *et al.* 1987, Ruiz-Hubard *et al.* 1987, McKentry 1990). NiTi rotary files lead to significant less debris extrusion compared to hand instrumentation techniques (Bidar *et al.* 2004, Kustarci *et al.* 2008a, Kustarci *et al.* 2008b). The results from recent studies including the comparison of debris extrusion among different NiTi rotary systems (Tanalp *et al.* 2006, Kustarci *et al.* 2008c, Logani & Shah 2008, Madhusudhana *et al.* 2010, Tasdemir *et al.* 2010, Froughreyhani *et al.* 2011), reciprocating files or between the two of them (Bürklein & Schäfer 2012, Bürklein *et al.* 2013, Koçak *et al.* 2013) are controversial.

The existing evidence state that clinicians should consider that all instrumentation and irrigation techniques induce debris extrusion. The aforementioned quantitative data cannot be directly correlated with clinical conditions. The selection of appropriate techniques that minimise debris extrusion in relationship to the periapical status may potentially reduce the risk of the emerging side-effects post-operatively. From a clinical point of view, apical extrusion in root canal procedures may predispose for the occurrence of inter-appointment flare ups, post-operative pain and delayed healing response (Siqueira 2003, Ng *et al.* 2008). However, apart from the quantity and quality of the extruded content, it is still unclear whether or how the level of the host's immune resistance is associated and affected in cases of apical extrusion.

1.16 Chemical interaction of NaOCl with other root canal irrigants

Several studies have shown that the use of a combination of NaOCl and EDTA (15-17%) is particularly effective in the removal of organic and inorganic debris (Goldman *et al.* 1982, Yamada *et al.* 1983, Baumgartner & Mader 1987). On a chemical basis, the antagonistic effects

between NaOCl and EDTA have been thoroughly investigated, when used as root canal irrigants. In many studies, the residual free available chlorine content of NaOCl solutions was measured with iodometric titrations when mixed with EDTA at different ratios and time intervals. Theirs results concluded that due to an unspecified chemical reaction the free available chlorine content is significantly reduced; therefore, the direct mixture, or the intermittent use was not indicated due to the upcoming limited antimicrobial capacity of NaOCl (Krishnan *et al.* 2017, Clarkson *et al.* 2011).

The application of solutions including NaOCl mixed with EDTA have showed to cause significant reduction in the capacity to dissolve bovine pulp tissue (Irala *et al.* 2010). Similarly, the tissue dissolution effects of the interactions between NaOCl and EDTA were tested on porcine palatal mucosa by assessing the percentage of original tissue weight after different exposure periods up to 120min. The study suggested that NaOCl alone was substantially more efficient than the other the solutions containing NaOCl and EDTA at different concentration ratios (Grawehr *et al.* 2003).

Chlorine gas formation was also detectable and the risks of occupational exposure has been highlighted (Baumgartner & Ibay 1987, Irala *et al.* 2010, Prado *et al.* 2013). The use of nuclear magnetic resonance analysis also confirmed that a chemical reaction between NaOCl and EDTA occurs, leading to NaOCl deactivation, slow oxidation of EDTA and progressive formation of unknown by-products (Grande *et al.* 2006). The degradation and consequent deactivation of EDTA after its interaction with NaOCl is extremely slow, and, therefore, it does not compromise its clinical performance with respect to its chelating, smear layer removal and dentine softening effects (Saquy *et al.* 1994, Grawehr *et al.* 2003, Zehnder *et al.* 2005).

A suggested clinical protocol by Zehnder (2006) for optimizing root canal disinfection before obturation, consists of irrigation with NaOCl to dissolve the organic components, irrigation with EDTA to eliminate the inorganic components of the smear layer and final flush with CHX to increase the antimicrobial spectrum of activity and impart substantivity. The possible chemical interactions between NaOCl and CHX gained significant attention due to the occuring acid-base reaction that takes place (Basrani *et al.* 2007). Some studies have reported the occurrence of a colour change and precipitation when NaOCl and CHX solutions are directly mixed (Vivacqua-

Gomes *et al.* 2002, Basrani *et al.* 2007). An initial concern has been raised that the colour change may have some clinical relevance because of staining and that the precipitate might interfere with the hermetic seal of the root filling (Vivacqua-Gomes *et al.* 2002). Bui *et al.* (2008) used environmental SEM and confirmed that a combination of NaOCl and CHX on root dentine left significant fewer patent dentinal tubules, compared with a negative control group.

Basrani *et al.* (2007) evaluated the chemical nature of this precipitate by using x-ray photon spectroscopy (XPS) and Time of Flight-secondary ion mass spectrometry (TOF-SIMS). The authors reported that an immediate reaction between 2% CHX and NaOCl, even at a low concentration (0.023%), occurred. The forming precipitate consisted mainly of para-chloroaniline (PCA) and the concentration directly increased with the increasing concentration of NaOCl. A study conducted by the same research group aimed to identify further the precipitate by using gas chromatography–mass spectrometry (GC-MS). The mixture of NaOCl 6% and CHX 2% resulted in the formation of PCA, without the presence of any other aniline derivatives or chlorobenzene (Basrani *et al.* 2010).

Toxicologic studies in animals have shown that the hematopoietic system is the major target for PCA toxicity, leading to formation of methemogloblin and the development of hemolytic anemia (Chhabra *et al.* 1991). Humans studies reported the incidence of severe methemoglobinemia in neonates exposed to PCA as a result of the breakdown of CHX to PCA by the humidifier heater in neonatal incubators (Hazardous Substances Data Bank, 2018).

Because of its possible toxic effect, it was advised that NaOCl should not be used in combination with CHX until more information on its effects on humans is available (Basrani *et al.* 2007, Basrani *et al.* 2010). Further investigations of the NaOCl/CHX precipitate are required to address the bioavailability and cytotoxicity of PCA that might be leached out of dentine subsequent to endodontic treatment.

The application of TOF-SIMS was proposed to study the formation of precipitate and PCA on the surface and in the tubules of dentine irrigated with NaOCl, followed either by EDTA, NaOCl, and CHX or by saline and QMiX (Dentsply Tulsa, USA) (Kolosowski *et al.* 2014). QMiX is a novel irrigation compound containing EDTA, CHX. As a non-specified detergent, it tends to decrease surface tension and increase surface wettability, as well as it combines antimicrobial activity and

capacity to remove the smear layer (Stojicic *et al.* 2012, Eliot *et al.* 2014). Standardised dentine blocks obtained from human teeth, were embedded in resin and cross-sectioned to expose dentine surface. The specimens were immersed in 2.5% NaOCl, followed by 17% EDTA, 2.5% NaOCl, and 2% or in 2.5% NaOCl, followed by saline and QMiX and subjected to TOF-SIMS spectra analysis. Irregular precipitate containing PCA and CHX breakdown products was formed in dentine irrigated with NaOCl followed by CHX, leading to occlusion and extension into the tubules. On the contrary, no precipitates or PCA were detected in the tubules of dentine irrigated with NaOCl followed by saline and QMiX (Kolosowski *et al.* 2014).

The same method was used by the study of Piperidou *et al.* (2018), in order to assess the formation of precipitate and PCA, when a combined irrigation of dentine with NaOCl and SmearOff (Vista Dental Products, USA) was performed. SmearOff is a similar product to QMix and contains EDTA and CHX. The analysis of treated dentine surfaces with TOF-SIMS and XPS disclosed the absence of precipitate or PCA formation, when SmearOff was combined with NaOCl, instead of EDTA and CHX.

An *ex vivo* irrigation tooth model was proposed by Arslan *et al.* (2015) to compare CHX and QMix in terms of orange-brown precipitate generation in root canals and analysis of PCA formation by mixing CHX and QMix with NaOCl. The root canals of sterile single-rooted teeth were initially prepared using rotary instruments and 2ml 2.5% NaOCl in every instrument change. The specimens were then divided and finally irrigated with distilled water (60s), 5ml of 2% CHX (60s) and 5ml of QMix (60s). The roots were split longitudinally, and the amount of orange-brown precipitate was evaluated using a stereomicroscope. To evaluate whether the precipitates included PCA, 2% CHX and QMix were separately mixed with 2.5% NaOCl in two flasks, at 1:1 volume ratio. After centrifugation, precipitates were obtained and analysed using 1^{H} nuclear magnetic resonance (NMR) spectra. CHX had significantly higher scores than QMix in terms of orange-brown precipitate formed in the root canals and precipitate chemical analysis disclosed that PCA was present in the mixture of CHX and NaOCl, but not in the mixture of QMix and NaOCl.

Despite the increasing concerns over the identification and formation of PCA following the interaction of NaOCl and CHX, several laboratory studies published controversial results. In two

published experimental set-ups, the researchers used one-dimensional (^{1}H) and two-dimensional (^{2}H) NMR spectroscopy (Thomas & Sem 2010, Nowiski & Sem 2011). According to their results, the reaction mixture of NaOCl and CHX did not produce PCA at any measurable quantity, and further investigation was indicated to determine the chemical composition of the brown precipitate (Thomas & Sem 2010). Nowiski & Sem (2011) identified two major CHX breakdown products, neither of which were PCA. Both products were related to PCA and were characterized as para-substituted benzene compounds. Based on NMR data and their proposed mechanism of CHX breakdown, the products were parachlorophenylurea and parachlorophenylguanidyl-1,6-diguanidyl-hexane. Therefore, along with native CHX, the precipitate contained two chemical fragments derived from CHX, neither of which were PCA.

A more recent study was designed to apply several analytical tools including high performance liquid chromatography (HPLC), ^{1}H- NMR spectroscopy, gas chromatography (GC), thin layer chromatography (TLC), infrared spectroscopy and GC-MS (Orban *et al.* 2016). To obtain a brown precipitate, 5% NaOCl was mixed with 2% CHX. This brown precipitate was analyzed and compared with signals obtained from commercially available 5% NaOCl, 2% CHX and 98% PCA in powder form. The results proved that the forming precipitate did not contain PCA when non-destructive methods such as ^{1}H-NMR, HPLC, GC and TLC were applied. On the contrary, the use of GC-MS is based on fragmentation of investigated structure. The detection of PCA may have been false positive, since it also appears to be present as a possible fragment of CHX in ionization process during mass analysis (Orban *et al.* 2016). The authors stated that this study will be a cutoff proof for the argument on PCA formation from reaction of CHX and NaOCl.

The combination of CHX and EDTA produces a white precipitate. Rasimick *et al.* (2008) determined if the precipitate involves the chemical degradation of CHX. The forming precipitate was re-dissolved in a known amount of dilute trifluoroacetic acid and the amount of CHX and EDTA was determined by reverse-phase HPLC with ultraviolet detection at 288nm. The results of chemical analysis showed that CHX forms a salt with EDTA rather than undergoing a chemical reaction, forming a salt (Rasimick *et al.* 2008).

1.17 NaOCl: Formation of disinfection by-products (DBPs) on water de-contamination procedures

NaOCl has been extensively used for the disinfection of drinking water, sewage-water plants, water supply and distribution systems, swimming pools and industrial applications. Chlorination is one of the most widely practised public health forms of disinfection in the developed world with the aim of preventing the spread of infection and contamination (Wallace *et al.* 2002). Proper chlorination kills majority of bacteria, viruses and parasites responsible for waterborne diseases (Chaitez *et al.* 2003).

Although water disinfection is essential for reducing the risk of pathogens in the public water supply, potentially harmful halogenated disinfection by-products (DBPs) can be formed by the reaction of chlorine with natural organic matter (NOM) and inorganic chemical precursors, such as halide ions including chlorite (Cl-), bromide (Br-), iodide (I-), nitrite (NO_2-) in the water (Nikolaou *et al.* 1999, Chen & Westerhoff 2010, Krasner 2009). The presence of DBPs in the domestic water supply results in widespread exposure from activities such as water consumption, bathing, showering, and swimming (Lin & Hoang 1999, Backer *et al.* 2000, Ashley *et al.* 2005, Chowdhury *et al.* 2014).

1.17.1 Classification of DBPs

Several categories of by-products are currently under investigation. Up-to-date, more than 300 species have been identified. The first and most widely studied group of DBPs was trihalomethanes (THMs) (Bellar *et al.* 1974, Rook 1974) followed by haloacetic acids (HAAs) (Quimby *et al.* 1980, Christman *et al.* 1983, Miller & Uden 1983, Reckow & Singer 1984, Krasner *et al.* 1989). Other prevalent categories of DBPs are haloaldehydes, haloketones, haloacetonitriles, chloropicrin and chlorophenols (Nikolaou *et al.* 1999, Krasner *et al.* 1989, Williams *et al.* 1997, Golfinopoulos & Nikolaou 2005) (Table 1-2).

Table 1-2 Classification and chemical type of most prevalent chlorinated disinfection by-products (DBPs).

Nomenclature of chlorinated disinfection by-products (DBPs)	**Chemical type**
Trihalomethanes (THMs)	
Chloroform	$CHCl_3$
Haloacetic Acids (HAAs)	
Chloroacetic acid	$Cl\text{-}CH_2COOH$
Dichloroacetic acid	$Cl_2\text{-}CHCOOH$
Trichloroacetic acid	$Cl_3\text{-}CCOOH$
Haloaldehydes	
Chloroacetaldehyde	$Cl\text{-}CH_2CHO$
Dichloroacetaldehyde	$Cl_2\text{-}CHCHO$
Trichloroacetaldehyde	$Cl_3\text{-}CCHO$
Chloral hydrate	$C_2H_3Cl_3O_2$
Haloketones	
1,1-dichloroacetone	$C_2H_4Cl_2CO$
1,3-dichloroacetone	$C_2H_4Cl_2CO$
1,1,1-trichloroacetone	$C_2H_3Cl_3CO$
Haloacetonitriles	
Chloroacetonitrile	$Cl\text{-}CH_2CN$
Dichloroacetonitrile	$Cl_2\text{-}CHCN$
Trichloroacetonitrile	$Cl_3\text{-}CCN$
Chloropicrin	CCl_3NO_2
Chlorophenols	
2-, 3-, 4- chlorophenol	$Cl\text{-}C_6H_4OH$
,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5- dichlorophenol	$Cl_2\text{-}\ C_6H_3OH$
2,3,4-, 2,3,5-, 2,3,6-, 2,4,5-, 2,4,6-, 3,4,5- trichlorophenol	$Cl_3\text{-}\ C_6H_2OH$
pentachlorophenol	$Cl_5\text{-}C_6OH$

1.17.2 Factors that affect the formation of DBPs

The factors that lead to DBP formation are: Concentration of chlorine and residual chlorine, pH, contact/reaction time, temperature and properties of natural organic matter (NOM) (Krasner *et al.* 1989, Singer 1994, Pourmoghaddas & Stevens 1995, Lekkas 1996, Williams *et al.* 1997, LeBel *et al.* 1997).

With increasing chlorine dose and residual, formation of HAAs becomes greater than THM formation. Depletion of the free chlorine residual ceases THM and HAA formation. However, limited formation of some other DBPs continues due to occurring hydrolysis reactions (Singer 1994). With increasing pH, THM formation increases, whereas HAA formation decreases (Krasner *et al*. 1989, Pourmoghaddas & Stevens 1995). At high pH values, hydrolysis of many halogenated DBPs occurs (Krasner *et al.* 1989). With increasing contact time, THM and HAA formation increases. On the other hand, DBPs such as haloacetonitriles and haloketones, which were initially formed, decay as a result of hydrolysis and reactions with residual chlorine (Singer 1994). When temperature increases, reactions are faster and a higher chlorine dose is required, leading to higher formation of DBPs (Golfinopoulos *et al.* 1993). With increasing NOM concentration, DBP formation increases (Reckow *et al.* 1990).

Apart from concentration, the composition of NOM expressed as total dissolved organic carbon (DOC) is influential for the formation of DBPs (Krasner et al. 1989, Singer 1994, Pourmoghaddas & Stevens 1995, Lekkas 1996, Williams et al. 1997, LeBel et al. 1997).

Principal categories of NOM include allochthonous, in which domination of humic substances is evident, and autochthonous, which is microbially derived and non-humic organic matter dominates (Krasner *et al.* 1996). The emerging sources of NOM include algal organic matter (AOM) and waste-water effluent organic matter (EfOM) (Mitch *et al.* 1999). Both AOM and EfOM are microbial in origin and are sources of organic nitrogen. AOM can be of extracellular or intracellular origin and includes macromolecules and cell fragments. EfOM is composed of degradation products and soluble microbial products (SMPs) (Namkung & Rittman 1986, Barker & Stuckey 1999). SMPs consist of macromolecules and cellular debris with a protein (nitrogen-enriched) and polysaccharide marker. Aminoacids are an important part of the organic-nitrogen content in water (Dotson *et al.* 2008). Chlorination of aminoacids can result in the formation of

aldehydes and nitriles, with subsequent or concomitant chlorine substitution to form chloral hydrate (hydrolysed form of trichloroacetaldehyde) and dichloroacetonitrile, respectively (Trehy *et al.* 1986).

1.17.3 Health concerns, DBP regulation and safety guidelines

Since the detection of DBPs, there have been concerns regarding their harmful effects on human health (Krasner 2009). A 30-year summary of the potential cytotoxic and mutagenic effects of DBPs, that provide data and risk factors with regard to occurrence, genotoxicity, carcinogenicity and health concerns was highlighted by Richardson *et al.* (2007).

In addition, there is a wide range of epidemiological studies, reviews and meta-analyses which target in the risk assessment and the effects of exposure to DBPs on the occurrence of cancer (McGeehin *et al.* 1993, Villanueva et al. 2007, Cantor *et al.* 2010, Rahman *et al.* 2010), on fetal growth (Grellier *et al.* 2010), the development of congenital anomalies (Nieuwenhuijsen *et al.* 2008) and adverse effects in pregnancy (Nieuwenhuijsen *et al.* 2000).

As a consequence, in order to minimize the risk of cancers, research is being actively carried out by the United States Environmental Protection Agency (USEPA), World Health Organization (WHO) and the European Union (EU), which has led to the introduction of regulations for THMs in drinking water (Table 1-3). The existing guidelines represent the concentration of a constituent that does not result in any significant risk to health of consumer over a lifetime of consumption. To assure consumers that drinking water is safe and can be consumed without any risk, guidelines or standards have been set up giving maximum allowable concentrations of compound in drinking water.

Table 1-3 Classification of regulated disinfection by-products (DBPs) by USEPA, WHO, EU.

DBP REGULATION AND GUIDELINES	
U.S. EPA regulations [a]	**Maximum Contaminant Level (MCL) (mg/L)**
Total Trihalomethanes (THMs)	**0.080**
Five Haloacetic acids (5HAAs)	**0.060**
Chlorite	0.010
Bromate	1.0
World Health Organization (WHO) guidelines [b]	**Guideline Value (mg/L)**
Chloroform	**0.2**
Bromodichlomethane	0.06
Chlorodibromomethane	0.1
Bromoform	0.1
Dichloroacetic acid	**0.05***
Trichloroacetic acid	**0.2**
Bromate	0.01*
Chlorite	0.7*
Chloral Hydrate (trichloroacetaldehyde)	**0.01***
Dichloroacetonitrile	**0.02***
Dibromoacetonitrile	0.07
Cyanogen chloride	0.07
2,4,6- Trichlorophenol	0.2
Formaldehyde	0.9
European Union Standards [c]	**Standard Value (mg/L)**
Total THMs	**0.1**
Bromate	0.01**
[a] Stage 1 Disinfectants/DBP (D/DBP) Rule	

[b] http://www.who.int/water_sanitation_health-/dwq/gdwq3/en [c] http://www.nucfilm.com/eu_water_directive.pdf	
*provisional values **where possible without compromising disinfection, EU member states should strive for a lower value. This value must be met, at the latest, 10 calendar years after the issue of the directive(Nov 1998); within 5 years of the directive, a value of 25µg/L must be met	

1.17.4 Application of activated carbon (AC) for the removal of DBPs

The application of enhanced coagulation, ozonation and treatment with activated carbon (AC) are among the proposed scientific methods for the elimination of VOCs, DBPs and the reduction of NOM precursors, SMPs and humic substances (Lou *et al.* 2009, Liu & Li 2015, Zhang *et al.* 2017). The use of AC has been advocated for the removal of organic constituents, impurities and residual disinfectants in water supplies due to its adsorption capacity (Zhao *et al.* 2018).

AC is a porous carbonaceous material prepared through the carbonization and activation of organic substances, mainly of vegetable origin. During carbonization of raw lignocellulosic material, a solid residue (charcoal) and volatile gases are produced. AC is produced by pyrolysis of organic materials of plant origin. These materials include coal, coconut shells and wood, sugarcane bagasse, soybean hulls and nutshell (Dias *et al.* 2007, Paraskeva *et al*. 2008). During this process pores and voids between the graphite crystals, are formed. Activation occurs immediately after carbonization. Activation may be chemical or physical. The intermediate product is removed and the graphite crystals become exposed to the activating agent (Smíšek & Cerný 1970). This increases the number and the dimensions of the pores during activation.

AC's adsorptive properties are used to remove organics. Adsorption is a natural process by which molecules of a dissolved compound collect on and adhere to the surface of an adsorbent solid. The occurrence of adsorption is related to the generation of forces between molecules to adhere to each other. AC adsorbs organic material because the attractive forces between the carbon surface which is non-polar and a non-polar contaminant are stronger than the forces

keeping the contaminant dissolved in water, under conditions of higher polarity. The adsorptive forces are weak and cannot occur unless the organic molecules are close to the carbon's surface. The large contact surface of AC due to its particle size and mesopore configuration allows for the adsorption to take place. The specific capacity of activated carbon to adsorb organic compounds is related to: total contact time, the molecular surface attraction, the total surface area available per unit weight of carbon, and the concentration of contaminants in the wastewater stream.

AC can further remove or eliminate the concentrations of residual chlorinated disinfectants through a catalytic reduction reaction, that involves a transfer of electrons from the AC's surface to the residual disinfectant; therefore, AC acts as a reducing agent. Chlorine removal results from the reduction of hypochlorous acid to a non-oxidative chloride ion.

Despite its chemical versatility, AC does not effectively dissociate bacteria and viruses, which may adhere on the surface and mesopores, using the latter as carbon source for their growth and metabolism (Kim & Park 2008). For the prevention of pathogens' growth on the AC surface and to exert antimicrobial activity, silver-impregnated activated carbon (Ag-AC) has been proposed for the point-of-se (POU) treatment of water systems (Shimabuku *et al.* 2017).

1.17.5 Theoretical engagement of endodontic research and chemistry of DBPs

The application of NaOCl as a main irrigant promotes a wide range of chemical interactions with the multivariable content of the root canal system. The identity of the content is a mixture of disintegrated necrotic pulp tissue, bacteria and bacterial fragments, irrigants, infected or non-infected dentine debris and tissue fluids, which are representative of the formed smear layer during instrumentation (Torabinejad *et al.* 1985). Thus, a resource and a constant reservoir of NOM becomes available during the chemomechanical preparation of the root canal system. NaOCl decomposes organic tissue components, co-reacts with inorganic components and at the same time the effectiveness of NaOCl as a disinfectant is also compromised.

The study conducted by Varise *et al.* (2014) was the first to highlight the importance of the formation of organochlorine compounds and toxic by-products in association with the reaction of NaOCl and the organic matter inside the root canals. This study used GC-MS to determine what compounds were produced as a result of the contact of different concentrations of NaOCl with bovine dental pulp and dentine.

For analysis of the products formed in the volatile phase, bovine pulp tissue interacted with 0.5%, 2.5% and 5.25% NaOCl until complete tissue dissolution. Bovine dentine powder was also kept in contact with NaOCl. During both experimental procedures, a solid phase microextraction (SPME) fiber was exposed inside the container through the cover membrane and then immediately injected into the GC-MS system. The same protocol was used for the aqueous phase. For analysis of the volatile compounds, the final solution was extracted using pure ethyl ether. The aqueous and volatile phases of both chlorinated dentine and pulp tissues showed the formation of chloroform, hexachloroethane, dichloromethylbenzene and benzaldehyde.

From a qualitative chemical prospective, a parameter that has not been investigated yet is the examination of the constitution of the emerging chlorinated suspensions, during and after chemomechanical preparation of necrotic or infected root canals. The debris and smear layer formation can be considered as a post-chlorinated mixture of organic and inorganic content existing in a liquid or semi-liquid suspension during instrumentation or irrigation, respectively.

1.18 Alternative strategies for root canal disinfection: Application of nanoparticles (NPs)

In the present literature review, it has been well documented that despite the clinical effectiveness of NaOCl as the main root canal irrigant, the deleterious effects against dentine collagen and caustic effects against soft and hard tissues should not be underestimated (Pashley *et al.* 1985, Oyarzún *et al.* 2002, Kerbl *et al.* 2012). The formation of toxic volatile compounds and chlorinated disinfection by-products has also been reported, when NaOCl reacts with compounds of infected root canal content (Varise *et al.* 2014). Other agents that exhibit some advantages are

CHX and EDTA, however the ability to totally disrupt biofilms remain questionable (Yoshida *et al.*1995, Naenni *et al.* 2004).

To address biocompatibility issues, biofilm resistance within complex root canal anatomy and restrictions by factors like concentration, time and volume, it is essential to search for new endodontic biomaterials for the promotion of root canal disinfection. The incorporation of nanoparticles (NPs) to advocate novel disinfection strategies has gained attention in the past few decades due to their innovative and functional properties. Any type of biocompatible particles with dimensions of 5–350 nm in diameter can be defined as NPs (Abeylath & Turos 2008). These include spherical, cubic and needle-like nanoscaled particles (Cushing *et al.* 2004).

Nanoparticles have received considerable attention within a range of diverse fields - including medicine and dentistry- resulting in advanced biomedical applications such as drug delivery, tissue regeneration, antimicrobial application, gene transfection, and imaging (Cohen 2001, Cushing *et al.* 2004, Venugopal *et al.* 2008). In nanotechnology, a dimensional decrease up to the atomic level leads to a considerable increase in surface area of the agent. High surface areas and consequently higher concentrations at target site, are predominant factors for the antimicrobial effectiveness of NPs, when compared with their conventional counterparts, since the effective interaction with the membrane of pathogens increases (Sawai *et al.*1996, Jamamoto *et al.* 1998).

A recent literature review referred to the use of term ‘nanodentistry’, which defines the application of nanomaterials and dental nanorobots toward diagnosis and treatment, with the goal of improving comprehensive oral health (Shrestha & Kishen 2016). A wide variety of oral health–related issues such as treatment of dentine hypersensitivity, biofilm elimination, diagnosis and treatment of oral cancers, as well as bone replacement materials are included in the spectrum of nanoparticle use (Abou Neel *et al.* 2015). In the field of Endodontics, the development of nanomaterials is focused on steps that would improve antimicrobial efficacy, mechanical integrity of previously diseased dentine matrix and tissue regeneration. Currently, newer technologies are being tested in endodontics, mainly toward overcoming the microbial challenge (Abou Neel *et al.* 2015).

Various types of NPs have been proposed in endodontic literature including metal NPs and mainly silver (Ag) NPs, polymeric NPs such as chitosan and triclosan NPs, and bio-active inorganic NPs containing bio-active glass, zinc-oxide or calcium silicate NPs (Samiei *et al.* 2016). Silver nanoparticles (Ag-NPs) are relatively new and gained the most attention among the metals used for the development of NPs. Silver exhibits a strong antibacterial activity against a wide range of microorganisms, in planktonic form, such as *P. aeruginosa*, *E. coli*, *S. aureus* and *C. albic*ans (Birla *et al.* 2009, Kim *et al.* 2009). Silver nanoparticles interact with the bacterial cell membrane, increase permeability and prevent DNA replication (Rai *et al.* 2012, Samiei *et al.* 2016, Shrestha & Kishen 2016). As a different type of silver in a nano-scale level, with different chemical and physical properties, Ag-NPs present improved antibacterial activity compared with conventional silver-based materials (Rai *et al.* 2009).

The use of aqueous suspensions of Ag-NPs has been recently proposed in the field of Endodontology (Wu *et al.* 2014). However, the results of several *in vitro* studies were not promising or presented conflicting outcomes. A 0.1% Ag-NPs suspension was tested against NaOCl 2% in dentine sections, where a single- specie *E. faecalis* biofilm was grown. The irrigated sections were examined under CLSM and the 0.1% Ag-NP suspension displayed minor biofilm disruption capacity, compared to NaOCl (Wu *et al.* 2014). On the other hand, the biofilms treated with 0.02% Ag-NPs gel as a medicament for 7 days significantly eradicated the structure with the least number of residual viable *E. faecalis* cells in comparison with 0.01% Ag-NPs gel and calcium hydroxide groups (Wu *et al.* 2014). Afkhami *et al.* (2015) also concluded that the mixture of Ag-NPs with calcium hydroxide was effective as medicament against E*. faecalis*, in short-term medication (7 days).

Previous studies have further indicated that the rate of bacterial killing by NPs depended on the concentration and duration of interaction (Kishen *et al.* 2008, Shrestha *et al.* 2010, Zhuang *et al.* 2011). A latest study evaluated the antimicrobial action of an irrigant containing 94ppm Ag-NPs in an aqueous vehicle, NaOCl and CHX against *E. faecalis* biofilm and infected dentinal tubules of bovine dentine blocks (Rodriguez *et al.* 2018). The Ag-NPs solution eliminated fewer bacteria, but was able to dissolve more biofilm compared with CHX, whereas NaOCl had the greatest antimicrobial activity and biofilm dissolution capacity. The limited effectiveness of sole irrigation with Ag-NPs has been attributed to the resistance of the biofilm matrix and the short

time period of root canal irrigation (Rodriguez *et al.* 2018). Other studies have showed that sole Ag-NPs may not be stable in suspension to exert their antimicrobial activity due to agglomeration, resulting from the resistance of some bacteria, which can produce the adhesive flagellum protein flagellin, that triggers the aggregation of the Ag NPs (Panáček *et al.* 2018).

Carbon materials are known to be more environmentally and biologically friendly than inorganic materials, since the carbon is one of the most common elements in our ecosystem. Graphene oxide (GO) is an allotropic type of carbon and was first successfully prepared by Geim and Novoselov in 2004. It is chemically exfoliated from oxidized graphite and is constituted of a monolayer of carbon nanosheets that form dense honeycomb structures (Novoselov *et al.* 2004, Geim & Novoselov 2007). These contain hydroxyl and epoxide functional groups on the two sides and carboxylic groups at the edges (Li *et al.* 2008, Park & Ruoff 2009). GO has been reported to exhibit antibacterial activity against several bacterial species (Akhavan & Ghanderi 2010, Tu *et al.* 2013, He *et al.* 2015, Nanda *et al.* 2016) and is considered as a promising material for biological applications (Chung *et al.* 2013).

A desire to explore the combination of the unique antimicrobial and biomedical properties of Ag-NPs and GO led to several attempts for the synthesis of Ag-NPs on GO matrix (Das *et al.* 2011). It was recently confirmed that the formation of a hybrid biomaterial of Ag-NPs, where a layered material like GO is used as matrix, can induce binding capability, that usually lacks in Ag-NPs alone and thus enhance its antimicrobial activity (Liu *et al.* 2018). The prospective from the use of a formulation containing Ag-NPs synthesized on an aqueous GO matrix (Ag-GO) for endodontic irrigation of infected root canals has not been investigated yet.

1.19 Summary

The extrapolation of data from water, environment and chemistry research domains provides sufficient indications regarding the existing potential for DBP formation during the chlorination of the mechanically prepared root canals. The identification of DBPs in the root canal system and in the periapical space has not been elucidated yet. In addition, considering the volatility of DBPs, the production of aerosol spray in the pulp chamber and the aspiration of

contaminated chlorinated liquid suspensions from dental units to waste-water distribution systems, the need for the evaluation of the emergence of DBPs is mandatory.

The overwhelming amount of research data associated with the emergence of DBPs after the application of NaOCl and their potential to cause adverse effects in general health should be taken seriously into consideration. From that perspective, this project will possibly amend current concepts and theories during irrigation and instrumentation of the root canal system, with the addition of another potential parameter which is a risk factor in terms of health and safety concerns.

Hence, we will explore in-depth the effect of NaOCl concentration and volume on structural integrity of mineralised dentine, whilst measuring the efficacy of the NaOCl as irrigant in presence of organics (Chapter 2) (Figure 1-13). The second aspect of this thesis will be a systematic study on the generation of DBPs during root canal treatment (Chapters 3 & 4) (Figure 1-13). The third and final aspect of this study will explore methods to minimize the volume of DBPs formed that is shunted into waste water (Chapter 5), whilst developing an in situ method of removal of bacterial load within the canal using nano-carbon technology (Chapter 6) (Figure 1-13).

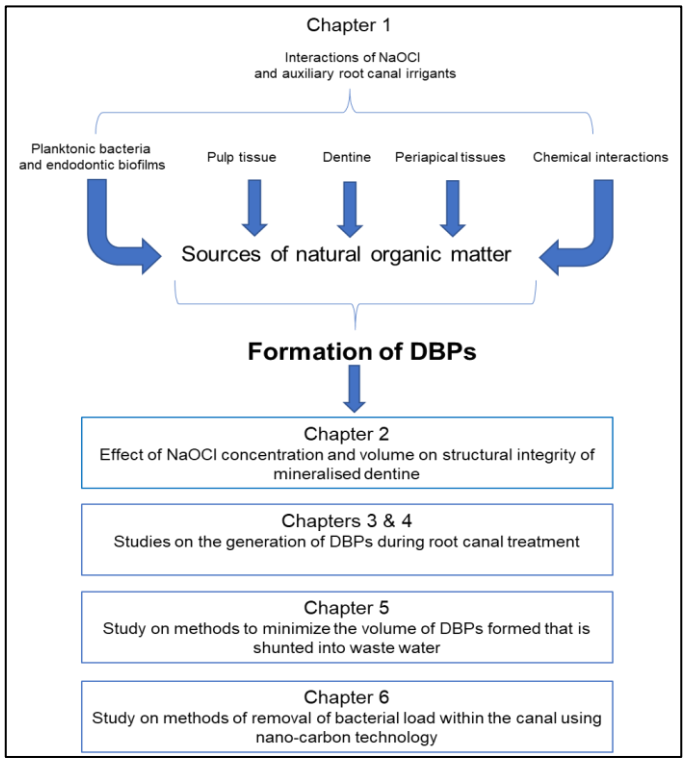


Figure 1-13 Flowchart of literature review data synthesis (Chapter 1), leading to the justification of potential formation of DBPs and step-by-step designation of experimental studies (Chapters 2-6).

Chapter 2

Proof of concept studies: The effect of NaOCl on structural integrity of dentine and formation of chlorinated DBPs

2.1 Introduction

A successful endodontic treatment outcome results from a combination of clinical procedures including mechanical preparation, irrigation, disinfection and three-dimensional obturation of the root canal system. The modern concept of root canal treatment considers mechanical preparation as a measure to facilitate delivery and exchange of the antimicrobial agents to the crucial apical root third (Gulabivala *et al.* 2010). NaOCl still remains gold standard for the irrigation and chemical disinfection of the root canal system. The proposed concentration in clinical use varies from 1.00-5.25% w/v in an attempt to balance antimicrobial and dissolution activity whilst considering its effects on cellular cytotoxicity, caustic effects and drawbacks on dentine's biomechanical properties (Sedgley *et al.* 2004).

During root canal irrigation, NaOCl interacts with radicular and coronal dentine whilst the irrigant flows in the root canal system or it remains deposited in the pulp chamber (Figure 2-1). One of the side effects of the interaction of NaOCl with dentine that has received relatively little attention in the endodontic literature is the impact on biomechanical properties (Oyarzun *et al.* 2002). A recent comprehensive review alluded to adverse biomechanical effects on properties of root dentine when NaOCl is used as an endodontic irrigant (Pascon *et al.* 2009). The effects of NaOCl on dentine micro-hardness (Saleh & Ettman 1999, Slutzky-Goldberg *et al.* 2004, Oliveira *et al.* 2007, Sayin *et al.* 2007), elastic modulus and flexural strength (Grigoratos *et al.* 2001, Sim *et al.* 2001, Marending *et al.* 2007) are time and concentration dependant.

NaOCl is a non-specific oxidizing and proteolytic agent, which oxidizes the organic matrix of dentine leading to denaturation of collagen components of the smear layer and alterations on structural integrity of mineralised dentine (Di Renzo *et al.* 2001, Oyarzun *et al.* 2002). The deproteinating effect of NaOCl on the organic structure of dentine has been documented in

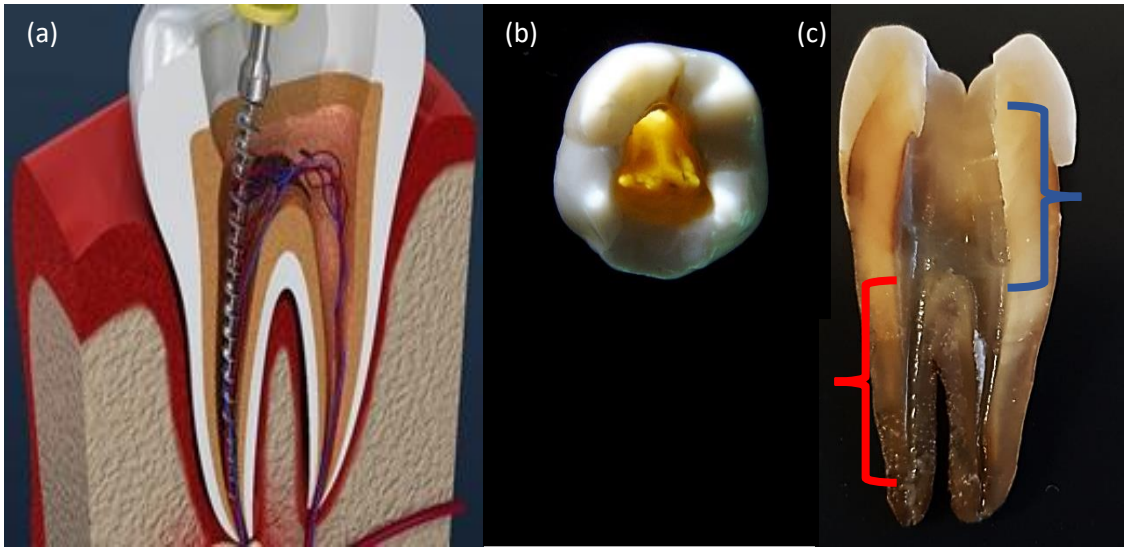


Figure 2-1 (a) Access cavity and instrumentation. (b) Pulp chamber and root canal orifices surrounded by coronal dentine (axial view). (c) Premolar coronal hemi-section depicting radicular dentine (red brace) and coronal dentine (blue brace) exposed to irrigants during root canal disinfection.

several studies. The results of imaging studies including SEM, TEM, polarised light microscopy (PLM) microscopy (Moreira *et al.* 2009, Zhang *et al.* 2010b), X-ray diffraction analysis (Ozdemir *et al.* 2012), immunohistochemistry (Oyarzun *et al.* 2002) and FTIR spectroscopy (Di Renzo *et al.* 2001, Hu *et al.* 2010, Zhang *et al.* 2010a) have shown that NaOCl interacts with the organic phase of dentine leading to concentration-dependent and time-dependent collagen depletion. On the contrary, NaOCl does not affect inorganic components of dentine (Hu *et al.* 2010, Ozdemir *et al.* 2012).

In a clinical setting, root canal preparation involves a variety of proposed instrumentation techniques, to optimize the efficacy of mechanical debridement and the delivery of irrigants to the entire root length (Hülsmann *et al.* 2005). An increase in root taper and apical size of preparation improves the flow rate and replenishment of NaOCl in the prepared root canal space (Boutsioukis *et al.* 2010a, Boutsioukis *et al.* 2010b). As mechanical preparation progresses, it is expected that the surface of the mechanically debrided root dentine is further exposed to increasing volumes of NaOCl; thus, the interaction of NaOCl with fresh dentinal organic and inorganic matter during chemo-mechanical preparation may result in gradual collagen degradation. On the other hand, an increase in treated dentine's surface or organic load may present an inhibitory effect on the protelolytic and oxidising action of NaOCl against collagen, due to loss of reactivity of chlorine (Macedo *et al.* 2010). Two major quantitative parameters that have not been examined yet are

the effects of dentine mass and irrigant volume on the alterations of organic and inorganic structure of mineralised dentine, treated by NaOCl.

One of the side effects of the interaction of NaOCl with dentine that has received relatively little attention in the endodontic literature is the formation of harmful chlorinated DBPs and additional chlorinated volatile hydrocarbons. Varise *et al.* (2014) reported the detection of several volatile organochlorine compounds, including chloroform, benzaldehyde, hexachloroethane, and dichloromethylbenzene following the 15-min interaction of NaOCl with bovine dentine powder and pulp tissue fragments with the aid of GC-MS. The application of head-space gas chromatography-mass spectrometry (head-space GC-MS) confirmed the formation of chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) after 60min of interaction (Ioannidis *et al.* 2016). The emergence of toxic chlorinated DBPs from the interaction of NaOCl with dentine requires further examination due to the arising potential hazardous drawbacks during root canal irrigation. To our knowledge, there are no quantitative reports on the formation of chlorinated DBPs in short and long exposure times in human dental tissue.

The aim of this study was to investigate the effect of commonly used NaOCl concentrations for root canal irrigation on the structural integrity of mineralised human dentine with Fourier Transform Infrared (FTIR) spectroscopy. To standardise the evaluation, different scales in mass of dentine in powdered form were used to enable comparison. The *ex vivo* comparison of the dentine mass and irrigant volume dependent effects of 1%, 2% and 5.25% NaOCl on the structural integrity of mineralised dentine was performed with ATR/FTIR spectroscopy. The study tested the 2-fold null hypothesis that the application of different NaOCl concentrations (1%, 2% and 5.25%) did not induce significant differences within organic and inorganic structure of mineralised dentine, associated with (1) dentine mass variations (5mg, 10mg, 15mg) or (2) irrigant volume variations (0.5ml, 0.3ml, 0.1ml). In addition, we screened and quantified *ex vivo* the formation of chlorinated DBPs resulting from the interaction of NaOCl 2.5% and 5% v/v with human dentine powder at several observation periods. The null hypothesis (Ho) was that NaOCl did not contain chlorinated DBPs and the interaction of NaOCl with human dentine did not result in the formation of chlorinated DBPs.

2.2 Materials and Methods

The proof of concept studies described here involve the demonstration of the oxidising effect of NaOCl against mineralised human dentine powder and the formation of chlorinated DBPs after analysing the aliquots obtained from the interaction of NaOCl with dentine. ATR-FTIR was used to determine the alterations in the IR spectra of treated dentine, whereas GC-MS was used to analyse the elicited volatile substances from their interaction.

2.2.1 The effect of dentine load, concentration and volume of NaOCl on structural integrity of mineralised dentine using ATR-FTIR spectroscopy.

2.2.1.1 Dentine preparation and treatment

Fifteen freshly extracted intact fully developed human impacted and semi-impacted mandibular third molars that were free of cracks, fractures, caries, abrasions and discolouration were collected. All procedures were approved and conducted in accordance with the protocol outlined by the Research Ethical Committee (Wales REC 4, 14/WA/1004, R&D Departiment, Dental Institute, Guy's Hospital, King's College, London, UK).

Access cavities were prepared to remove pulpal debris with stainless steel hand files and distilled water. The enamel and cementum layers were removed with high speed diamond conical burs (Dentsply International). The remaining dentine bulk was then reduced to a powder state by using low speed round burs (Dentsply International). Dentine powder particle size distribution was measured with a laser diffraction particle-size analyser (CILAS 1180, Orleans, France), which could analyse particles within the range 0.04-2500μm. Five samples of dentine powder (10mg) were randomly selected, inserted into a water tank and ultra-sonicated for 30s. Particle size distribution was expressed by the values obtained from the cumulative distributions with measures of central tendency (median diameter; d50) and distribution width (relative span = d90-d10 / d50).29 D50 values varied from 17.66μm to 31.03μm and relative span values varied from 1.10 to 2.62 units. The total sample of mineralised dentine powder was kept frozen at -80^0 C to prevent degradation of collagen components, until further use.

Different concentrations of NaOCl (1%, 2%, 5.25%) were obtained as readily available standard solutions for dental use (Chloraxid, Cerkamed, Poland). The total dentine powder sample was divided in different groups to study the effects of NaOCl concentration, dentine mass and irrigant volume on dentine. To study the effects of NaOCl of standard volume (0.5ml) on mineralised dentine powder of different weights (5mg, 10mg, 15mg), 3 experimental groups were formed (A; 1% NaOCl, B; 2% NaOCl, C; 5.25% NaOCl), and each of them consisted of 3 sub-groups (Table 2.1). To study the effects of different volumes (0.1ml, 0.3ml, 0.5ml) of NaOCl 1%, 2%, 5.25% w/v on mineralised dentine powder of standard weight (10mg), 3 experimental groups were formed (D; 1% NaOCl, E; 2% NaOCl, F; 5.25% NaOCl) and each of them consisted of 3 sub-groups (Table 2.2).

Distilled water served as a control group, to treat the variable weights of dentine powder (MDW) at different volumes (VDW) in the same manner as in the experimental groups A-F respectively.

The weights were measured on a balance (XS 105 Dual Range, Mettler Toledo). The volumetric measurements were carried out using pipettes scaled to 0.1-0.5ml pipette canisters (Gilson, UK). The interaction of dentine powders with the requisite solvents was carried out in 0.5ml-containing, conical-shaped, clear plastic test micro-tubes (StarLab UK Ltd). The micro-tubes were stabilised on a vertical axis and customised with the aid of silicone putty (Aquasil, Dentsply DeTrey, Germany) on plastic cylindrical moulds (7mm diameter – 3mm height). The mixes were placed on a platform shaker (Stuart Gyro-Rocker SSL3, Bibby Scientific Ltd, UK) and gently agitated for 5 min at 25rpm, followed by 1 min sonic irrigant activation with the aid of blue tip of Endo-Activator (Dentsply Maillefer, Switzerland) at top power (10000 cpm).

After treatment with NaOCl or DW, the dentine powders were washed with distilled water three times. The micro-tubes were subsequently sealed with the aid of their snap-cap and transferred to an Eppendorf centrifuge (Heraeus Fresco 21 Centrifuge, Thermo Scientific, Cole-Parmer, UK). The aliquots were centrifuged for 2min at 200 rpm at 22^0 C, to allow sedimentation of the powder. Distilled water was aspirated from the centrifuge tubes with a micropipette (Gilson, UK) and the dentine powder sediments were allowed to air-dry for 48h at ambient room temperature.

Table 2-1 Group allocation to study the effect of different concentrations of NaOCl at a fixed volume (0.5ml) on varying amount of dentine powder.

Groups	Sub-Groups	Weight of dentine (mg)
Group A (1% NaOCl)	A1	5
	A2	10
	A3	15
Group B (2% NaOCl)	B1	5
	B2	10
	B3	15
Group C (5.25% NaOCl)	C1	5
	C2	10
	C3	15
Control Group Distilled water (MDW)	MDW1	5
	MDW2	10
	MDW3	15

Table 2-2 Group allocation to study the effect of varying volume and concentrations of NaOCl on a fixed amount of dentine powder (10mg).

Groups	Sub-Groups	Volume of irrigant (ml)
Group D (1% NaOCl)	D1	0.5
	D2	0.3
	D3	0.1
Group E (2% NaOCl)	E1	0.5
	E2	0.3
	E3	0.1
Group F (5.25% NaOCl)	F1	0.5
	F2	0.3
	F3	0.1
Control Group Distilled water (MDW)	VDW1	0.5
	VDW2	0.3
	VDW3	0.1

2.2.1.2 ATR / FTIR analysis

A Fourier Transform Infrared Spectroscopy (FTIR) spectrometer with single reflection attenuated total reflection (ATR) set-up (Spectrum One, Perkin Elmer Inc, UK) was used to obtain infrared spectra from the dentinal 'superficial sub-surface' (Zhang *et al.* 2010) (Figure 2-2). Dentine powder was transferred and fitted to the aperture of a 2mm-diameter sampling window which covered the bottom surface of the diamond crystal which is incorporated in ATR set-up. Spectra were obtained between 650 and 4000 cm^{-1} at 4 cm^{-1} resolution, by using 32 scans and expressed as absorption. Between sampling procedures, the sampling the crystal surface and

aperture were cleaned with acetone. The infrared spectra of dentine powder were collected in triplicates.

Figure 2-2 (a) Transfer of treated dentine powder to the FTIR spectrometer (b) and fit into the aperture of the sampling window. (c) Production of laser beam to obtain IR spectra.

Full-range automatic baseline correction was performed after each measurement to correct spectra without distorting the band intensities and to remove baseline features caused by scattering. Spectra were further normalised to the most intense band in order to eliminate the path length variation and reduce the differences between each single measurement of the same sample. Because apatite is insoluble in NaOCl, the phosphate stretch vibration peak provided a constant intensity for the purpose of normalisation (Di Renzo *et al.* 2001). After baseline correction and normalisation, the effects of NaOCl on the organic and inorganic content of mineralised dentine powder were examined.

The amide I peak (C=O stretching vibration at 1600-1700cm^{-1}) is a sensitive indicator of the collagen component in dentine (Eliades *et al.* 1997, Di Renzo *et al.* 2001). The phosphate v_3 peak (v_3 PO_4^{3-} stretching vibration at 1020cm^{-1}) and carbonate v_2 peak (v_2 CO_3^{-2} stretching vibration at 870cm^{-1}) from 900 to 1200 cm^{-1} are assigned to the apatite component (Eliades *et al.* 1997, Di Renzo *et al.* 2001).

The peak intensity of the three bands was defined as the corrected peak area ($A.cm^{-1}$) which is the area between the spectrum and the linear marked baseline, within two base points (Amide I: Base 1=1720cm^{-1} & Base 2=1580cm^{-1}; Phosphate v_3: Base 1=1180cm^{-1} & Base 2=890cm^{-1} ; Carbonate v_2: Base 1=890cm^{-1} & Base 2=820cm^{-1}). The peak intensities were mathematically calculated with the incorporated software, and phosphate/amide ratio (P/A) and phosphate/carbonate ratio (P/C) were further calculated to examine the effects on organic and inorganic structure of mineralised dentine.

2.2.1.3 Statistical analysis

The dependent variables of this study were the 'amide I' (A), 'phosphate' (P) and 'carbonate' (C) intensity peaks as well as the 'phosphate/amide' (P/A ratio) and 'phosphate/carbonate' (P/C ratio) ratios. The independent variables were 'NaOCl concentration', 'dentine powder weight' and 'NaOCl volume'. To ensure the normal data distribution and homogeneity of variance for both experimental procedures, inverse transformation was performed for the dependent variables A, P and C. Two-way ANOVA with repeated measurements was used for data analysis. The significant effects and interactions of the experimental factors were investigated with pairwise comparisons, which were conducted with post hoc multiple comparisons and Bonferroni's correction.

The dependent variables P/A ratio and P/C ratio did not fulfil the normality and homoscedasticity assumptions; thus, the data were analysed with non-parametric Kruskal-Wallis test for independent samples. The overall analysis was performed with SPSS software (version 22.0, IBM SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P<0.05$.

2.2.2 *Ex vivo* investigation of NaOCl-dentine interaction and their effect on the formation of toxic chlorinated disinfection by-products (DBPs)

2.2.2.1 Dentine preparation and treatment

Tooth specimens were reduced to dentine powder in the same way as described in Chapter 2, section 2.2.1.1. The total dentine powder sample, 700mg, was equally divided in seven groups, each one containing 100mg. The weights were measured on a balance (XS 105 Dual Range, Mettler Toledo, Leicester, UK) and powder was kept at -80^0 C to prevent degradation of collagen components, until further use.

2.2.2.2 Characterisation of root canal irrigants 2.5% and 5% v/v NaOCl

In this section, we decided to prepare the NaOCl solutions, to ensure that no impurities or additives were present. The available chlorine content of a 10-15% v/v stock solution of NaOCl

(Sigma Aldrich, Gillingham, UK) was verified with a standard iodine/thiosulfate method (Vogel, 1962). The chemical reactants used were: (i) Sodium thiosulphate solution (0.2M) which was prepared from 50 g sodium thiosulphate pentahydrate crystals (99.8% purity, Alfa Aesar, Heysham, UK) dissolved in 1L HPLC water (Chromasolv, Sigma Aldrich, Gillingham, UK), and 25 mg of sodium carbonate (VWR, UK) were added to adjust the pH of the solution to alkaline levels, (ii) potassium iodate (0.05M) (Sigma Aldrich, Gillingham, UK) and potassium iodide (0.24M) (Alfa Aesar, Heysham, UK), (iii) sulphuric acid (3M) (Fisher Scientific, Loughborough, UK) and (iv) potato starch (2% w/v) (VWR, Lutterworth, UK).

Titrations (n=3) of sodium thiosulphate consumption were carried out after the standardisation of the titrating agent with potassium iodate (0.05M) as a primary standard at 0.22M. The concentration of NaOCl stock solution was found to be 1.474M. Stock NaOCl solution was diluted with HPLC water to obtain 5% v/v NaOCl (0.68M) and 2.5% v/v NaOCl (0.34M). The NaOCl solutions were stored in air-tight dark containers at 5^{o}C, until further use.

2.2.2.3 Interaction of NaOCl and dentine

To study the effect of NaOCl on dentine, aqueous suspensions of NaOCl 2.5% v/v (Group A; n=3), 5% v/v (Group B; n=3) and HPLC water (Group C-control; n=1) were individually subjected into 50ml-containing Erlenmeyer conical glass flasks. Every flask contained 100mg of dentine powder and 50ml of the experimental and control irrigants (Groups A-C). The samples were stirred with magnetic bars at 250 rpm, at ambient room temperature, to ensure homogeneous contact between irrigants and dentine powder.

Sampling procedures were performed in triplicates, at different time intervals: 30sec, 60sec, 90sec, 3, 10, 30, 60 and 90min. The aliquots were kept sealed in 1.5ml-containing microcentrifuge tubes. Blank solutions of 2.5% and 5% v/v NaOCl and HPLC water were equally collected. All samples were stored at -80^{0} C until analysis. At the day of analysis, samples were thawed at room temperature, centrifuged (2min at 200 rpm) and the aliquots were subjected to analysis.

The concentration of the study compounds with time was mathematically attempted to adjust into first- and second- order reactions, linearised expressions for which are given in equations (1) and (2) respectively. A first-order reaction (equation 1) is a reaction that proceeds at a rate that depends linearly on only one reactant concentration (a unimolecular reaction). A

second-order reaction (equation 2) is a type of chemical reaction that depends on the concentrations of one second-order reactant or two first-order reactants. This reaction proceeds at a rate proportional to the square of the concentration of one reactant, or the product of the concentrations of two reactants. In these equations, k is the velocity constant of the reaction and the starting and instantaneous concentrations of both study DBPs are expressed as $[DBP]_0$ and [DBPs], respectively.

$$\mathrm{Ln}[\mathrm{DBP}] = \ln[\mathrm{DBP}]_0 - k\,\mathrm{t} \qquad \text{Equation (1)}$$

$$\frac{1}{[DBP]} = \frac{1}{[DBP]o} + k\,t \qquad \text{Equation (2)}$$

2.2.2.4 Determination of DBPs with GC-MS

The analysis of potential DBP formation was performed by gas chromatography-mass spectrometry. The sample extracts were injected using 3 independent runs. The volatile fraction of the sample extracts was analysed with GC-MS (Model 7890, Agilent Technologies, Santa Clara, US), where the analyser was a quadrupole and the ionisation source was electron impact (70 eV) (GC-EI-MS) (Figure 2-3).

Figure 2-3 The GC-EI-MS analytical instrument Agilent 7890 (adopted from www.agilent.com).

Chromatographic separation was carried out with a column 30m x 0.25mm I.D of 0.25µm of film thickness BPX5 fused-silica capillary column with stationary phase 5% phenyl polysilphenylene-siloxane (SGE Analytical Science, UK). The samples (2µL) were injected with split 1:10 and the temperature of the injector was 220ºC. Single ion monitoring acquisition mode (SIM) was used to acquire 2 ions from each analyte, according the findings of a previous pilot study (Ioannidis *et al.* 2016). For $CHCl_3$, m/z 83 and 85, were monitored with dwell time 20 ms, and the ions corresponding to CCl_4, m/z 117 and 119, were monitored with 80 ms dwell time.

The separation program started at 40 ºC, temperature which was held for 5 min and increased to 220 ºC at 36 ºC·min-1. Under these conditions, the limit of detection for, assessed at a signal-to-noise ratio of 3, with the SIM mode was 0.5 and 8 µg/L for $CHCl_3$ and CCl_4, respectively. An overview of the volatile fraction was carried out in full scan mode, with acquisition ranges m/z 50-150 and 150-300. The separation conditions in full scan acquisition mode were 40ºC for 5 min to 220 ºC at 5 ºC·min-1 and held at 220 ºC for 5 min. Both chromatographic separations were carried out with He at 1ml·min^{-1}. NIST 08 standard reference database was the library used to identify unknown compounds in the samples (National Institute of Standards and Technology, Gaithersburg, US).

2.2.2.5 Statistical analysis

The dependent variables of this study were the concentrations (mg/L) of $CHCl_3$ and CCl_4. The independent variables were the type of NaOCl solutions (2.5% v/v and 5.0% v/v) and sampling time intervals (30s-90min) from the interaction of NaOCl and mineralised human dentine powder. The dependent variables did not fulfil the normality and homoscedasticity assumptions; thus, the data were analysed with non-parametric Kruskal-Wallis test for independent samples. The overall analysis was performed with SPSS software (version 22.0, IBM SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P<0.05$.

2.3 Results and discussion

2.3.1 The effect of dentine load, concentration and volume of NaOCl on structural integrity of mineralised dentine using Fourier Transform Infrared Spectroscopy.

In this study, the effects of NaOCl on dentine were quantitatively analysed with the aid of ATR/ FTIR. FTIR spectroscopy is an effective, direct, non-destructive and sensitive analytical technique for the identification of organic and inorganic materials. The FTIR technique measures the absorption of various infrared light wavelengths by the material under examination. The infrared absorption bands identify specific molecular components and structures and are highly specific for each material.

The infrared absorption frequencies for native dentine have been extensively investigated (Di Renzo *et al.* 2001, Eliadis *et al.*1997). Thus, any spectral changes caused by alteration in dentine collagen structure and inorganic hydroxyapatite can be therefore compared to reference values for the assessment of changes that a treatment may have induced (Di Renzo *et al.* 2001, Eliadis *et al.*1997). Mineralised human dentine was reduced to a powder state and the FTIR spectra recorded, represented the 'superficial sub-surface', since the depth of penetration of evanescent light is limited in ATR/FTIR (Zhang *et al.* 2010b); thus, the acquired information can be representative of variations occurring in the organic and inorganic phase of dentine within the bulk (Zhang *et al.* 2010b). Since the interaction of the dentine powder and irrigants were carried out in bulk, it is anticipated that the reaction occurs within the entire powder mass. Triplicate samples were used from the bulk that should further confirm the findings.

The normalization of the peaks is a process that minimizes potential errors due to specimen variations in thickness, size or contact surface of the sample, as well as differences in masses employed to prepare a sample. In our study, we took every effort to minimise such probability. For the quantification of the peak intensities, we intended to identify peaks deriving from bonds which were not affected by the oxidising effect caused by the action of NaOCl. The signal obtained by the phosphate stretch vibration peak was employed since it does not change appreciably. As a result, the interpretation of the spectra and the conclusions drawn were not affected by the selection of phosphate stretch vibration peak for normalisation.

Two different experimental procedures were performed with a view to investigate whether variations in mineralised dentine powder weight and irrigant volume were contributory or posed any inhibitory action towards NaOCl effects in the organic and inorganic content of mineralised dentine powder. A typical ATR/FTIR spectrum of intact human dentine powder without NaOCl treatment is shown in Figure 2-4. Typical peak assignments are shown in the spectrum, which is in agreement with literature findings (Eliades *et al.* 1997, Di Renzo *et al.* 2001, Mountouris *et al.* 2004).

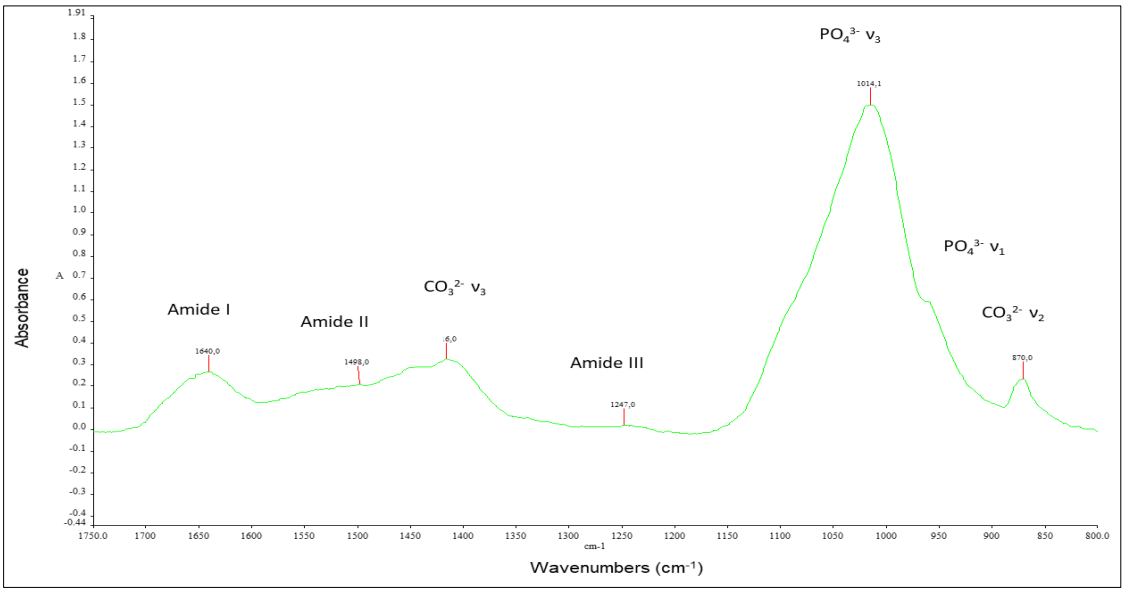


Figure 2-4 A typical ATR spectrum of human dentine without NaOCl treatment.

2.3.1.1 Interaction of NaOCl with dentine powder and the effect of weight variations on A, P, C intensity peaks, P/A and P/C ratios.

After treatment with NaOCl, the normalised spectra clearly showed a reduction in intensity of the peaks at 1242, 1550, 1643 cm^{-1}, regardless of NaOCl concentration and weight of dentine powder. In addition, the presence of phosphate and carbonate peaks became more apparent in comparison to the control group (Figure 2-5).

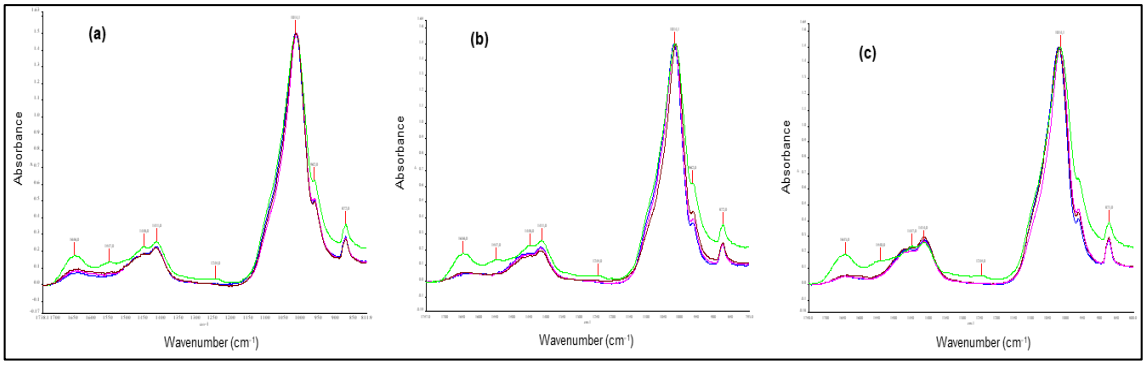


Figure 2-5 ATR spectra of human dentine powder treated by (a) NaOCl 1%; (b) NaOCl 2%; (c) NaOCl 5.25% (**Green**: DW; **Blue**: 5mg dentine powder; **Rose**: 10mg dentine powder; **Brown**: 15mg dentine powder).

The results of mean (SD) values of A, P, C intensity peaks, P/A and P/C ratios for Groups A, B, C, control group (MDW) and their sub-groups are presented in Table 2.3. The mean(SD) values of Amide I intensity peaks of experimental groups A, B, C were significantly reduced compared to control group (MDW) ($P<0.05$). When NaOCl 1% (Group A) and 2% (Group B) were used, the mean(SD) values of A intensity peaks were proportional to dentine powder weight. An increase in dentine powder weight was associated with higher mean(SD) values of Amide I intensity peaks in Groups A and B ($P<0.05$). NaOCl concentration and the interaction with dentine weight did not affect the mean(SD) values of Phosphate and Carbonate intensity peaks.

The mean (SD) values of P/A ratios among experimental groups A, B, C, control group (MDW) and their sub-groups respectively are presented in Table 2-3. The distribution of P/A ratios in Groups A, B, C were statistically significantly different in comparison to control group (MDW) ($P<0.05$). In Groups A and B, the distribution of P/A ratios between sub-groups presented statistically significant differences (A_1 vs A_2 vs A_3, $P<0.05$), (B_1 vs B_2 vs B_3, $P<0.05$) and mean values decreased as dentine powder weight increased from sub-group A_1 to A_3 and B_1 to B_3, respectively. In Group C, the distribution of P/A ratio was the same across sub-groups and no statistically significant differences were observed (C_1 vs C_2 vs C_3, $p=0.156$).

The mean (SD) values of P/C ratios among experimental groups A, B, C, control group (MDW) and their sub-groups respectively are presented in Table 2-3. The distribution of P/C ratios of Groups A, B, C and control group (DW) did not present statistically significant differences ($p=0.912$).

Table 2-3 Mean (SD) values of Amide I, Phosphate and Carbonate intensity peaks, P/A and P/C ratios for experimental Groups A, B, C, control group (MDW) and their sub-groups.

	Dentine weight (mg)	**AMIDE I (A) Intensity (1640 cm^{-1}) Mean(SD)**	**PHOSPHATE (P) Intensity (1014 cm^{-1}) Mean(SD)**	**CARBONATE (C) Intensity (870 cm^{-1}) Mean(SD)**	**P/A RATIO Mean(SD)**	**P/C RATIO Mean(SD)**
GROUP A NAOCl 1%						
A1	*5*	2.9 (0.3) *	127.2 (3.1)	2.4 (0.1)	44.1 (2.9) *	52.5 (1.2)
A2	*10*	3.3 (0.2) *	127.0 (2.9)	2.4 (0.1)	38.2 (2.2) *	53.9 (2.5)
A3	*15*	3.9 (0.1) *	126.8 (0.9)	2.3 (0.1)	32.6 (0.5) *	55.2 (2.0)
GROUP B NAOCl 2%						
B1.	*5*	1.7 (0.1) **	121.8 (2.1)	2.3 (0.07)	73.1 (3.5) **	52.6 (1.7)
B2.	*10*	2.0 (0.1) **	123.0 (3.0)	2.2 (0.07)	60.7 (2.4) **	52.6 (1.5)
B3	*15*	2.1 (0.1) **	118.3 (2.2)	2.9 (0.2)	56.6 (1.8) **	53.9 (1.2)
GROUP C NAOCl 5.25%						
C1.	*5*	2.0 (0.1)	124.6 (2.9)	2.9 (0.2)	61.6 (3.0)	42.5 (2.3)
C2.	*10*	2.0 (0.1)	123.4 (3.2)	2.8 (0.1)	62.6 (1.8)	43.5 (1.1)
C3.	*15*	2.1 (0.1)	123.5 (3.4)	3.2 (0.02)	59.5 (1.2)	38.8 (1.0)
CONTROL GROUP Distilled water						
MDW1.	*5*	7.3 (0.2)	131.5 (0.8)	2.4 (0.1)	17.9 (0.5)	54.7 (1.7)
MDW2.	*10*	7.3 (0.1)	132.0 (0.9)	2.6 (0.2)	18.1 (0.3)	50.3 (3.4)
MDW3.	*15*	7.3 (0.1)	132.2 (1.1)	2.6 (0.2)	18.0 (0.4)	51.8 (3.7)

*: Sub-groups A 1-3; statistically significant differences within sub-group comparisons (P<0.05)

**: Sub-groups B 1-3; statistically significant differences within sub-group comparisons (P<0.05)

2.3.1.2 Interaction of NaOCl with dentine powder and the effects of irrigant volume alterations on Amide I, Phosphate, Carbonate intensity peaks, P/A and P/C ratio

After treatment with NaOCl, the normalised spectra clearly showed a reduction in intensity of the peaks at 1242, 1550, 1643 cm^{-1}, regardless of concentration and irrigant volume. In addition, the presence of phosphate and carbonate peaks became more apparent in comparison to the control group (Figure 2-6).

The results of mean (SD) values of Amide I, Phosphate and Carbonate intensity peaks, P/A and P/C ratios for Groups D, E, F, control group (VDW) and their sub-groups are presented in Table 2.4. When NaOCl 1% (Group A) and 2% (Group B) were used, the mean(SD) values of Amide I intensity peaks were in inverse proportion to irrigant volume. An increase in the volume

of reacting NaOCl 1% and 2% with dentine powder of standard weight was associated with lower mean(SD) values of Amide I intensity peaks in Groups D and E (P<0.05). NaOCl concentration and volume alterations did not affect the mean(SD) values of Phosphate and Carbonate intensity peaks.

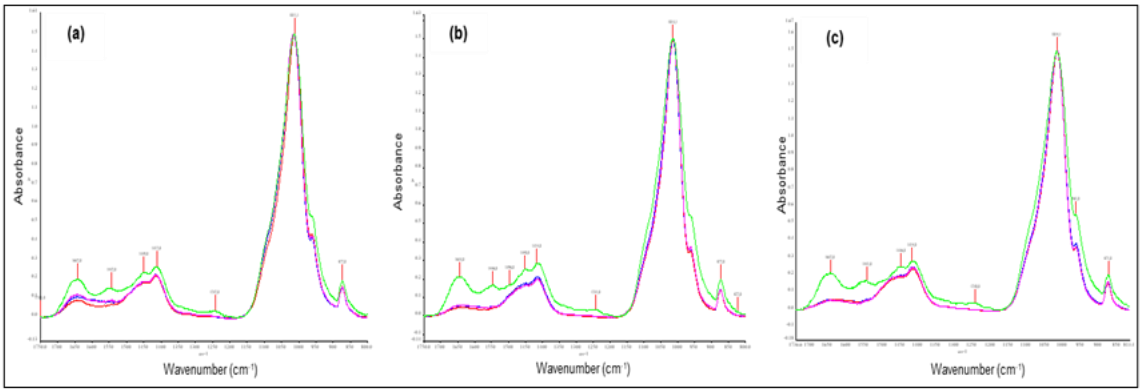


Figure 2-6 ATR spectra of human dentine powder treated by a. NaOCl 1%; b. NaOCl 2%; c. NaoCl 5.25% (**Green**: DW; **Red**: 0.5mL NaOCl; **Blue**: 0.3mL NaOCl; **Rose**: 0.1mL NaOCl).

The mean (SD) values of P/A ratios among experimental groups D, E, F, control group (VDW) and their sub-groups respectively are presented in Table 2-4. The distribution of P/A ratios in Groups D, E, F were statistically significantly different in comparison to control group (VDW) (P<0.05). In Groups D and E, the distribution of P/A ratios between sub-groups presented statistically significant differences (D_1 vs D_2 vs D_3, P<0.05), (E_1 vs E_2 vs E_3, P<0.05) and mean values decreased as irrigant volume decreased from sub-group D_1 to D_3 and E_1 to E_3, respectively. In Group F, the distribution of P/A ratio was the same across sub-groups and no statistically significant differences were observed (F_1 vs F_2 vs F_3, P=0.403).

The mean (SD) values of P/C ratios among experimental groups D, E, F, control group (VDW) and their sub-groups respectively are presented in Table 2-4. The distribution of P/C ratios of Groups D, E, F and control group (VDW) did not present statistically significant differences (P=0.960).

Table 2-4 Mean (SD) values of Amide I, Phosphate and Carbonate intensity peaks, P/A and P/C ratios for experimental Groups D, E, F, control group (VDW) and their sub-groups.

	Irrigant volume (ml)	AMIDE I (A) Intensity (1640 cm^{-1}) Mean(SD)	PHOSPHATE (P) Intensity (1014 cm^{-1}) Mean(SD)	CARBONATE (C) Intensity (870 cm^{-1}) Mean(SD)	P/A RATIO Mean(SD)	P/C RATIO Mean(SD)
GROUP D NAOCL 1%						
D1	*0.5*	3.9 (0.1) *	131.2 (2.3)	2.5 (0.1)	34.1 (1.0) **	53.1 (1.0)
D2	*0.3*	4.4 (0.2) *	133.3 (1.6)	2.6 (0.1)	30.4 (1.2) **	52.1 (1.2)
D3	0.1	5.0 (0.3) *	133.1 (2.3)	2.5 (0.1)	26.7 (1.0) **	54.0 (1.8)
GROUP E NAOCL 2%						
E1.	*0.5*	2.1 (0.2) *	123.9 (1.8)	2.3 (0.1)	59.4 (4.6) **	53.4 (1.8)
E2.	*0.3*	2.4 (0.1) *	123.4 (2.0)	2.2 (0.1)	52.0 (1.1) **	54.9 (0.8)
E3.	0.1	3.2 (0.4) *	130.4 (5.7)	2.5 (0.2)	41.0 (2.9) **	53.0 (1.9)
GROUP F NAOCL 5.25%						
F1	*0.5*	2.3 (0.1)	123.0 (2.3)	2.7 (0.04)	53.0 (1.6)	45.9 (0.4)
F2	*0.3*	2.4 (0.1)	125.7 (1.4)	2.7 (0.1)	52.8 (2.7)	46.6 (1.9)
F3	0.1	2.5 (0.2)	127.9 (3.5)	2.8 (0.1)	51.3 (2.5)	45.3 (1.3)
CONTROL GROUP (VDW)						
VDW1.	*0.5*	8.9 (0.3)	140.9 (1.3)	2.6 (0.2)	15.8 (0.6)	53.7 (4.2)
VDW2.	*0.3*	8.8 (0.3)	140.5 (1.9)	2.7 (0.1)	16.1 (0.4)	52.0 (1.0)
VDW3.	0.1	8.7 (0.2)	139.6 (2.2)	2.7 (0.1)	16.1 (0.2)	51.7 (2.1)

*: Sub-groups D 1-3; statistically significant differences within sub-group comparisons (P<0.05)
**: Sub-groups E 1-3; statistically significant differences within sub-group comparisons (P<0.05)

2.3.1.3 NaOCl concentration-related effects on dentine structure

The results of this study showed that NaOCl had deleterious effects on the organic components but did not affect the inorganic phase of human mineralised dentine powder. A concentration-related effect was also evident, leading to P/A ratios increase as irrigant concentration increased, respectively. The increase in P/A ratio is attributed to the subsequent reduction of Amide I intensity (Hu *et al.* 2010, Zhang *et al.* 2010a, Zhang *et al.* 2010b). More specifically, an increase of dentine powder weight from 5mg towards 10 and 15mg had an inhibitory effect on collagen depletion when NaOCl 1% and NaOCl 2% were used. A reduction of the volume of delivered irrigant from 0.5ml towards 0.3 and 0.1ml had an inhibitory effect on collagen depletion when NaOCl 1% and NaOCl 2% were used. Equally, P/A ratios decreased as Amide I intensity remained less affected and presented higher values when a larger quantity of dentine weight or smaller irrigant volume was applied.

The delivery of low volumes of irrigant and the presence of large contact surface with dentine may somewhat protect the organic phase of dentine. NaOCl interacts with dentine resulting in a loss of total available chlorine (Macedo *et al.* 2010). The loss of available chlorine in low NaOCl concentrations is faster than in high concentrations; thus, a quick loss of reactivity may not be detrimental for the structure of dentine organic matrix (Macedo *et al.* 2010).

On the contrary, the effect of NaOCl 5.25% on mineralised human dentine powder organic phase was not inhibited neither by alterations in dentine weight nor by alterations in delivered irrigant volume. The mean (SD) values of Amide I intensity remained unchanged at low levels, whereas the mean (SD) values of P/A ratios remained unchanged at high levels due to collagen depletion. The above findings suggest that the oxidative potential of NaOCl 5.25% against dentine is not affected by inhibitory parameters such as reduced irrigant volume, increased quantity of organic matter and the protective encapsulation of collagen into apatite crystals.

NaOCl did not substantially affect the inorganic phase of mineralised dentine powder. Although IR spectra of phosphate peaks became more apparent in comparison to control group, the distribution of P/C ratios did not present differences between the experimental and control groups. These findings are in agreement a previous study (Hu *et al.* 2010). However, mean (SD) values of P/C ratios in NaOCl 5.25% (groups C and F) were lower than the rest experimental and control groups. It has been reported that high concentrations of NaOCl can remove not only the organic content but also carbonate and magnesium ions from dentine crystal structure as well (Sakae *et al.*1988).

The delivery of low volumes of irrigant and the presence of large contact surface with dentine may somewhat protect the organic phase of dentine. NaOCl interacts with dentine resulting in a loss of total available chlorine (Macedo *et al.* 2010). The loss of available chlorine in low NaOCl concentrations is faster than in high concentrations; thus, a quick loss of reactivity may not be detrimental for the structure of dentine organic matrix (Macedo *et al.* 2010).

2.3.1.4 NaOCl time-related effects on dentine structure

Mineralised human dentine powder was treated with NaOCl and distilled water solutions for a total period of 6 minutes. This period is realistic time proportionate to clinical conditions as it corresponds to the time required for instrument sequencing, intermediate irrigation and sonic

activation during chemomechanical preparation. Hu *et al.* (2010) reported that short exposure times (between 1 and 10 min) did not produce significant differences in dentine deproteination induced by 0.5%, 1.0% and 2.25% NaOCl solutions.

Dentine treatment with higher NaOCl concentrations (5%, 12%) can lead to dramatic decrease of amide:phosphate ratio within 100-120s (Di Renzo *et al.* 2001, Mountouris *et al.* 2004). This could be explained by the fact that the collagen exposed on the dentin surfaces can be quickly dissolved and depleted by NaOCl. The deproteination process of NaOCl may further revert to the hydroxyapatite-encapsulated collagen, which is protected by apatite crystals and does not present substantial change over a period of several minutes (Mountouris *et al.* 2004). The existing findings imply that the extent of deproteination caused by NaOCl is not linearly related to the increase in exposure time from 10s to several minutes (Di Renzo *et al.* 2001, Hu *et al.* 2010).

General agreement regarding the optimal concentration and duration of NaOCl irrigation does not exist. However, it would be prudent to outweigh and balance between antimicrobial activity and drawbacks of NaOCl against dentine biomechanical properties, even at short-term exposure times. Destruction of the collagen matrix and creation of an apatite-rich subsurface in dentine and mineralised tissues results in a less tough, more brittle substrate that might precipitate fatigue crack propagation during cyclic stresses (Kruzic & Ritchie 2008). This might increase the susceptibility of the root-treated teeth to post-treatment crown or root fracture (Pascon *et al.* 2009). Furthermore, the effect of chemical irrigation for endodontic purposes on dentine has further implications in restorative procedures. The application of NaOCl 5% adversely affects dentine bonding and bond strength leading to increased adhesive failures between the resin and dentine interface (Nikaido *et al.* 1999, Stevens 2014). NaOCl 5% also influences the sealing ability and the adhesion of resin-based cements and root canal sealers (Shinohara et al. 2004, Nassar *et al.* 2011). Furthermore, the deproteination of root canal dentine with NaOCl 5% leads to reduction of the push-out bond strength between dentine surfaces and cemented fiber posts (da Cunha *et al.* 2010, Saraiva *et al.* 2013).

From a clinical point of view, NaOCl 1% and 2% should be applied for routine use in an attempt to minimise dentine deproteination. Irrigant replenishment and delivery in high volumes may compensate for any inhibitory activity of the organic load towards their antimicrobial effectiveness. NaOCl 5.25% should be applied with caution in cases of persisting endodontic

infections, where endodontic pathogens may harbour within dentinal tubules. In such cases, the remaining dentine structure, the presence of prognostic factors associated with risk of tooth fracture and the restorative treatment plan should also be considered upon proper selection of NaOCl concentration.

2.3.2 *Ex vivo* investigation of NaOCl-dentine interaction and their effect on the formation of toxic chlorinated DBPs.

In this study, the application of GC-EI-MS analysis was essential for the identification of DBPs formed in post-chlorination aliquots, following the interaction of human mineralised dentine powder with NaOCl. GC-EI-MS is an analytical instrumental method, comprising a gas chromatograph coupled to a mass spectrometer, by which complex mixtures of chemicals may be separated, identified and quantified. Gas chromatography is used to separate mixtures into individual components using a temperature-controlled capillary column. The application of EI is a common method for ion production and a beam of electrons ionises the sample molecules resulting in the loss of one electron. MS is used to identify the various components from their mass spectra and can be compared with mass spectral databases, identified and quantified with the aid of internal standards. Several alternative techniques are available for the determination of chlorinated DBPs including GC-MS equipped with ECD (electron-capture detector) (Wang *et al.* 2013), SPME GC-MS (solid-phase microextraction) (Varise *et al.* 2014), head-space GC-MS (Ioannidis *et al.* 2016). The interaction of NaOCl with mineralised human dentine powder was previously examined by our group in a pilot study. The application of head-space gas chromatography-mass spectrometry (head-space GC-MS) confirmed the formation of chloroform and carbon tetrachloride after 60min of interaction (Ioannidis *et al.* 2016). Therefore, this experiment is a continuation of a previous work but with a different GC-MS instrument, in order to automate the increasing scale of number of experimental measurements required.

Full scan with narrow m/z ranges was used to keep an open view for the yield a range of volatile compounds at temperatures up to 220ºC. Such species were only detected in low abundancy in the first part of the chromatogram in NaOCl solutions. The spectra of NaOCl solutions which had been in contact with dentine showed changes in the levels of $CHCl_3$ and CCl_4,

which were quantified with a more sensitive and selective mode (SIM). No species where detected in the acquisition with scan range m/z 150-300, therefore, volatile compounds with long hydrocarbon chain (MW>150g/mol) and higher boiling point were not observed.

GC-MS analysis did not reveal detectable organic chlorinated compounds in ultrapure water and control ultrapure water samples incubated with mineralised human dentine powder (Group C).

2.3.2.1 Quantification of $CHCl_3$ and CCl_4 in NaOCl extracts

The analysis of $CHCl_3$ and CCl_4 was carried out with SIM mode of the ions m/z 83 and 85 and 117 and 119, respectively. The estimated limits of detection, assessed at a signal-to-noise ratio of 3, with the SIM mode was 0.5 and 8 µg/L for $CHCl_3$ and CCl_4, respectively. The working range used in the quantification of these compounds was 0.08-5 mg/L. A typical example of the separation and sensitivity obtained when injecting a standard is given in Figure 2-7. An example of calibration curves obtained after the use of $CHCl_3$ and CCl_4 standards is provided for a day of analysis ($CHCl_3$ calibration curve y = 280464x + 53127, R^2 = 0.9925; CCl_4 calibration curve y = 29237x – 2966.5, R^2 = 0.9936).

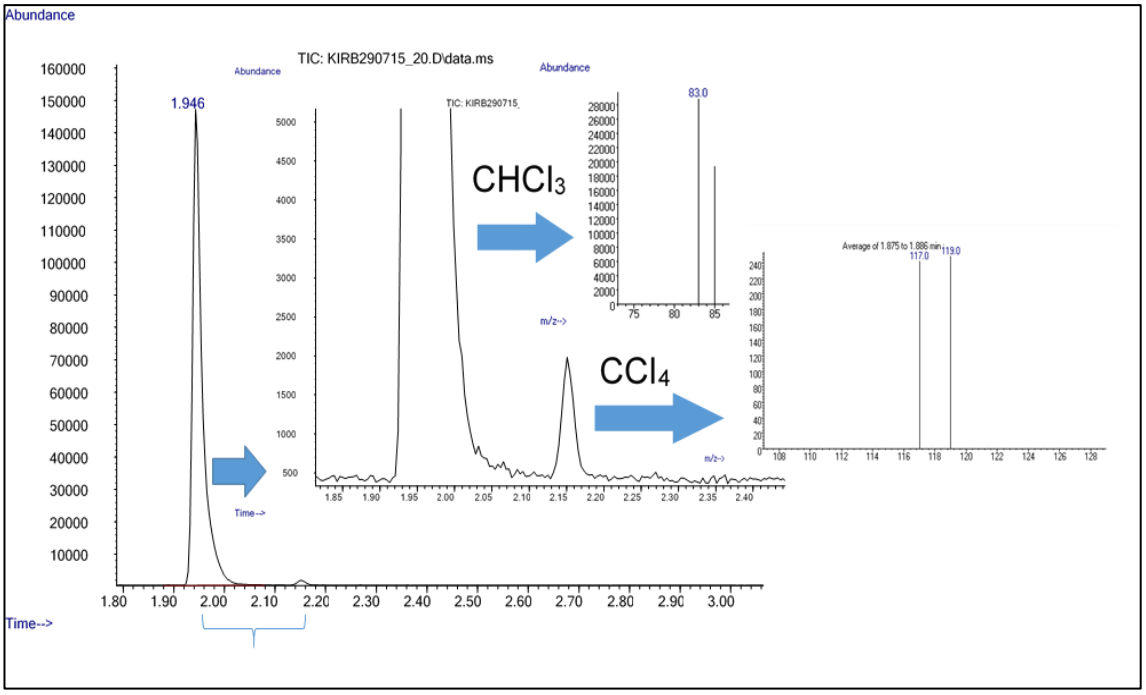


Figure 2-7 Chromatograph and spectra obtained after injection of internal standards 79µg/L $CHCl_3$ (peak retention time 1.946min) and 89µg/L CCl_4 (peak retention time 2.16min) in methanol.

The quantification of the study DBPs with GC-MS in the starting NaOCl solutions disclosed the presence of $CHCl_3$ and CCl_4 (Figure 2-8). On the contrary, the two compounds were not detected in HPLC water blank and HPLC water that had been in contact with dentine at all exposure times.

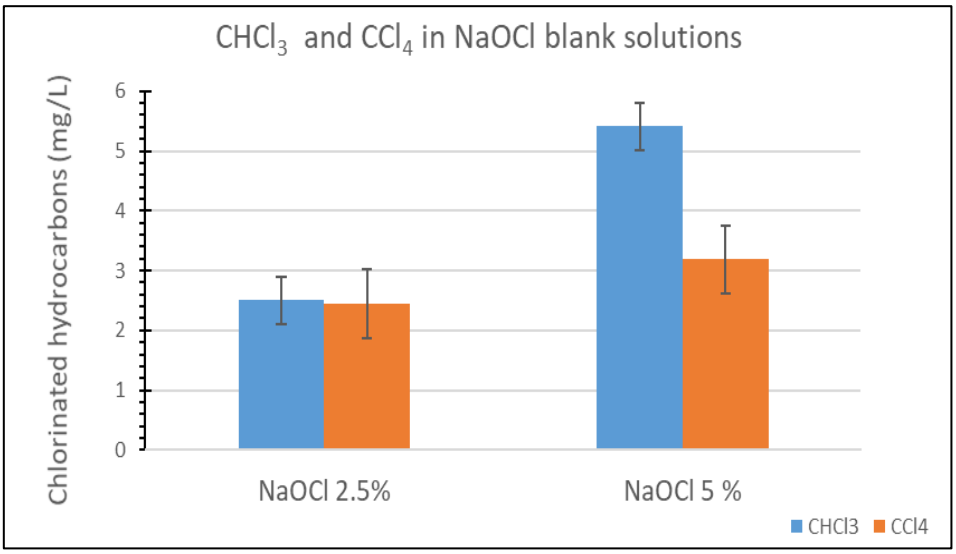


Figure 2-8 Determination of the two chlorinated hydrocarbon compounds in blank NaOCl solutions (no incubation with mineralised human dentine). Error bars correspond to the standard deviation from triplicate samples.

The levels of $CHCl_3$ or CCl_4 for triplicate dentine samples used in the kinetic study led to different concentrations over time and cannot be considered as triplicates according to non-parametric Kruskal-Wallis analysis. $CHCl_3$ and CCl_4 were present at all exposure times, their distribution in Groups A and B was equal and no statistically significant differences were observed ($P>0.05$).

The different concentration of $CHCl_3$ and CCl_4 over time with 2.5% and 5% NaOCl irrigant solution are shown in Figure 2-9, respectively. It can be observed that there are sharp increases in the concentration of $CHCl_3$, especially, and CCl_4, within the first minute. Following, after about 10 min there is a second rise in the concentration of the study compounds. The maximum concentrations of $CHCl_3$ and CCl_4 were 14.1 mg/l and 10.8 mg/l, respectively when the solution was NaOCl 2.5%. The profile of the quantified $CHCl_3$ at NaOCl 5% showed a similar trend compared to NaOCl 2.5% and the maximum yield of $CHCl_3$ was detected at 60min with a concentration of 15.9 mg/l. In contrast to $CHCl_3$, CCl_4 did not show great variations with time and

its concentration stayed within 0.5 and 2.3 mg/l. These concentrations are lower than the starting concentration and therefore a statistical increase was not observed in the concentration of CCl_4.

The changes in concentrations of $CHCl_3$ and CCl_4 followed different trends, which suggest that there may be different pathways leading to their production, besides possible losses due to their volatility or adsorption. Despite the existing heterogeneity of the acquired data, an attempt was made to characterise the occurring order of reaction of the samples analysed. The order of reaction refers to the power dependence of the rate on the concentration of each reactant (Bagshaw 2013). The order of reaction is an experimentally determined parameter and can take on a fractional value. This is distinct from the molecularity (or stoichiometry) of the reaction which is the theoretical integer value of the number of molecules involved in the reaction (Bagshaw 2013). Characterisation of the order of reaction was achieved particularly for Group A. The changes in concentrations adjusted well (> 0.98) to second-order kinetic reaction (equation 2) for the first 5min for CCl_4, and for 60min for $CHCl_3$ (Figure 2-10).

The mechanism of action and the complexity of the occurring oxidation reaction can be attributed to: (i) The existence of a reservoir of DBP compounds as pre-cursors in blank irrigant NaOCl solutions, (ii) the high concentration of NaOCl solutions and the increased chlorine reactivity rate after 90min of interaction (Macedo *et al.* 2010), (iv) the quick interaction with dentine as a source of NOM and the subsequent degradation of the organic content leading to collagen depletion (Hu *et al.* 2010), (v) the high degree of volatility of $CHCl_3$ and CCl_4 and the probability of constant release in gaseous form from the aqueous phase (Varise *et al.* 2014).

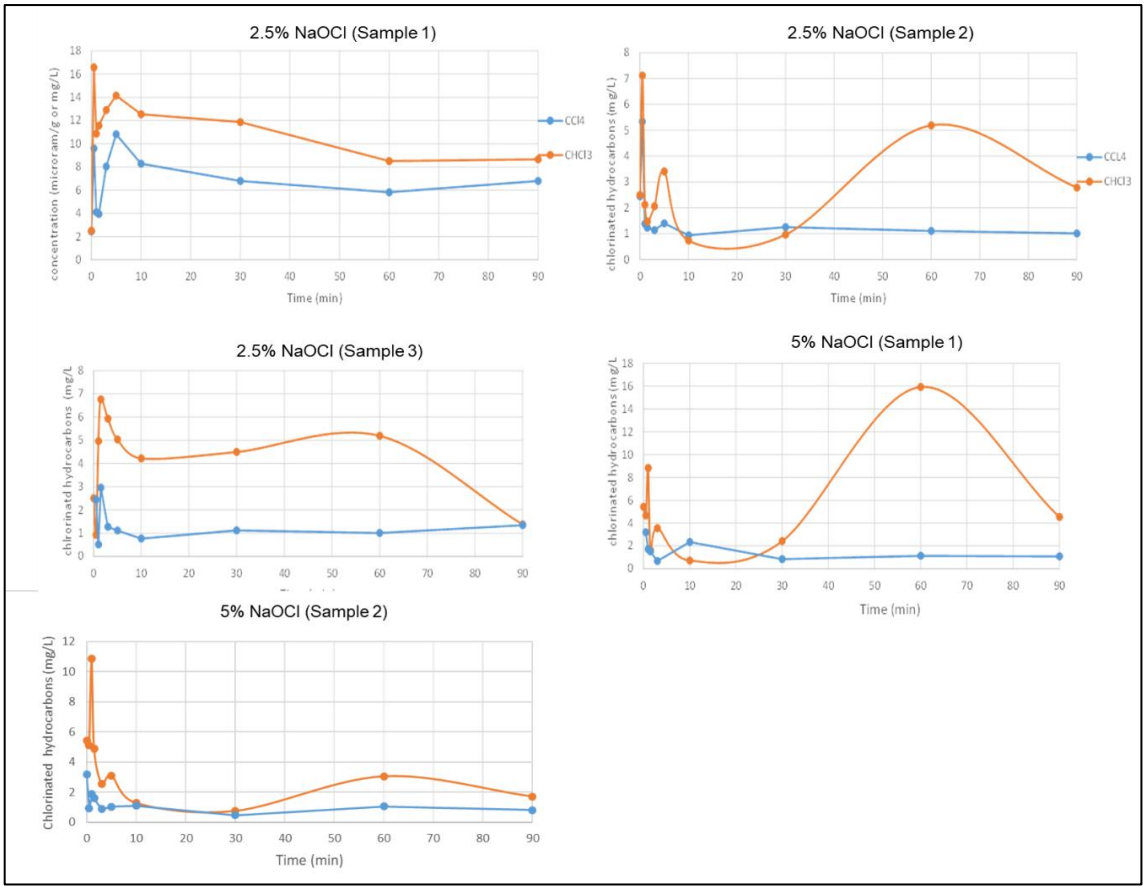


Figure 2-9 Comparison of the change in carbon tetrachloride (in blue) and chloroform (in orange) in triplicate samples (S1-S3, 2.5% NaOCl) and in duplicate (S1-S2, 5% NaOCl) samples. This was due to experimental problems arising during the analysis of the samples S3-5% NaOCl several times.

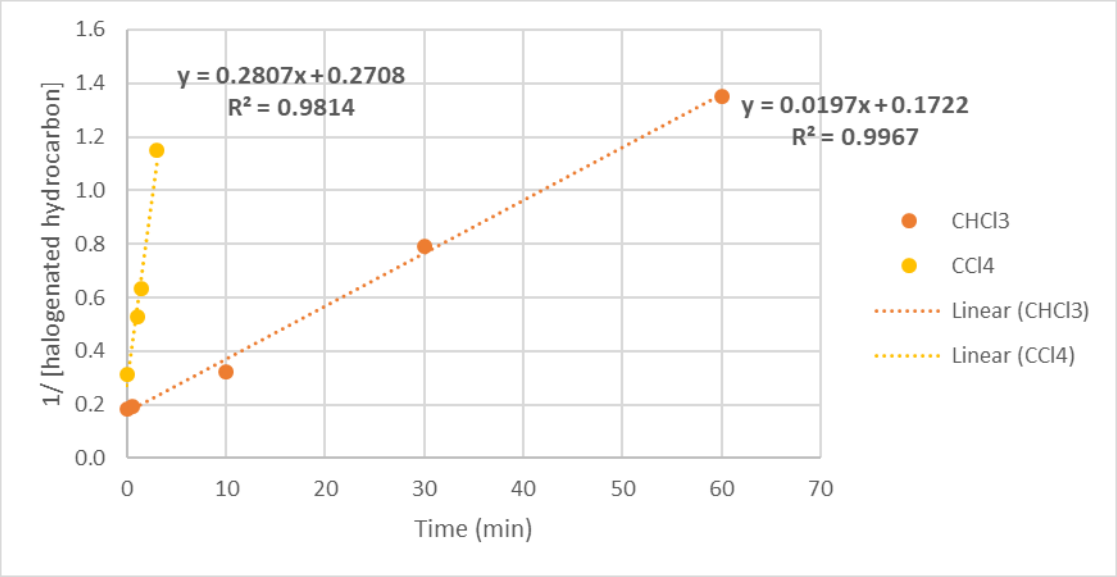


Figure 2-10 Changes in the concentration of $CHCl_3$ and CCl_4 with time in a stirred system containing dentine (100 mg) and 2.5% NaOCl (50 ml) adjusted to a second order kinetic model.

2.3.2.2 Search for unknown compounds

The identification of unknown chlorinated compounds in full scan acquisition mode was performed with the use of spectral libraries based on the full scan spectra and the boiling point of the suggested compounds by the library database. GC-MS analysis did not reveal detectable organic chlorinated compounds in ultrapure water and control ultrapure water samples incubated with mineralised human dentine powder (Group C).

When the scan range corresponded to ions m/z 50-150, the results of the extraction of the aqueous phase showed that blank NaOCl as well as the contact of NaOCl with mineralised human dentine powder resulted in the formation of a peak with retention time (RT) varying from 3.3min (blank NaOCl) to 3.5min (NaOCl+dentine) and mass to charge ratio (m/z=50.70.83) (Figure 2-11). The peak corresponded to the presence of bis(chloromethyl)ether (BCME) ($C_2H_4Cl_2O$) [IUPAC name: dichloro(dichloromethoxy)methane, MW =114 g/mol, boiling point=106.1°C].

No species where detected in the acquisition with scan range m/z 150-300 and compounds with long hydrocarbon chain (MW>150g/mol) and higher boiling point were not observed.

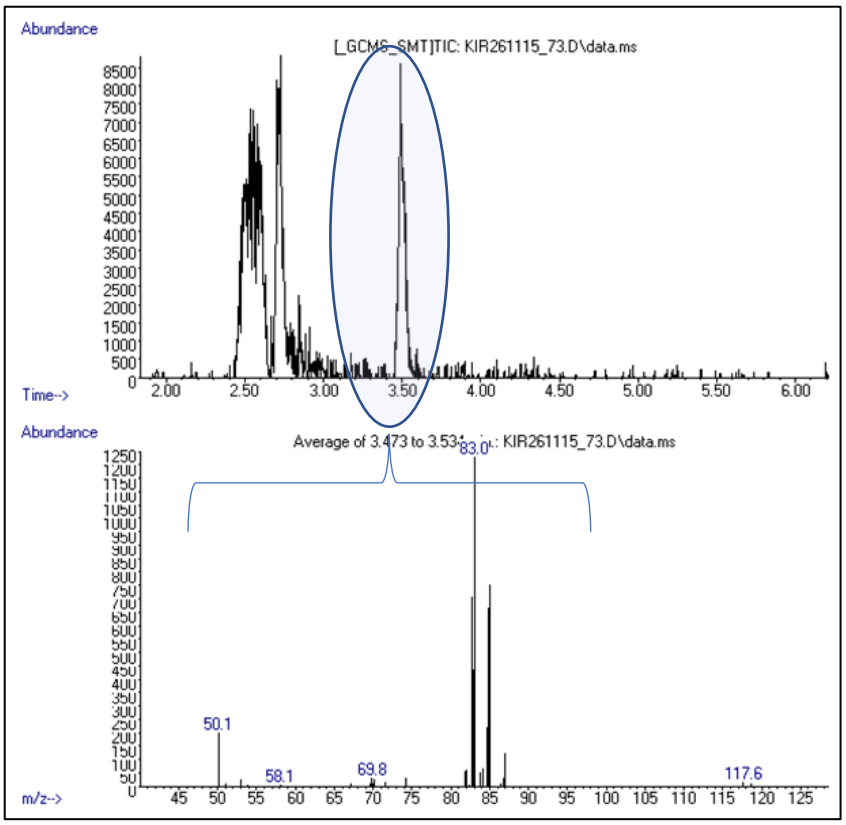


Figure 2-11 An example of chromatogram from the total ion current of ions m/z 50-150 from extract from dentine incubated with 2.5% NaOCl for 60 min. Spectra from the specie at 3.5 minutes is given. The libraries suggest that this peak could correspond to dichloro(dichloromethoxy)methane (IUPAC name), which has MW 184 g/mol and boiling point 106.1°C.

2.3.2.3 Chemical and biological implications

The chemical interaction of NaOCl with mineralised human dentine powder showed that DBPs remained detectable at all time intervals, though presenting different patterns of reaction. Despite the performance of the experimental procedures in triplicates under same laboratory conditions, the available data for the quantification of $CHCl_3$ and CCl_4 formation did not follow normal distribution. The formation of chlorinated DBPs was neither NaOCl concentration-related and nor time-related. The dissimilar levels of volatile DBPs in independent systems where dentine is stirred in NaOCl could further result from a different degree of loss through volatilisation of the study compounds or adsorption onto dentine due to their relatively low polarity. The obtained chromatographs from full scan acquisition mode, presented a constant increase in intensity of the peak of BCME with time.

Chloroform ($CHCl_3$) belongs to the category of chlorinated DBPs named as trihalomethanes (Bellar *et al.* 1974, Rook 1974). Chloroform has been shown to be carcinogenic in animals after oral exposure, resulting in an increase in kidney and liver tumours (ATSDR 1997). The United States Environmental Protection Agency (USEPA) has classified chloroform as a probable human carcinogen and is ranked in Group B2 (USEPA 1999). World Health Organisation (WHO) has concluded that the weight of evidence suggests that chloroform does not have direct genotoxic potential (WHO 2004).

Carbon tetrachloride (CCl_4) was produced in large quantities to produce chlorofluorocarbon refrigerants and propellants for aerosol cans, but consumer and fumigant uses have been discontinued and only industrial uses remain (ATSDR 2005). Studies in animals have shown that ingestion of carbon tetrachloride increases the risk of liver cancer. Decreased fertility and degenerative changes have been observed in animals exposed to carbon tetrachloride by inhalation (U.S. Environmental Protection Agency 2010). Human data on the carcinogenic effects of carbon tetrachloride are limited. USEPA has classified carbon tetrachloride as a Group B2, probable human carcinogen (U.S. Environmental Protection Agency 2010). There is sufficient evidence that carbon tetrachloride is carcinogenic in laboratory animals, but inadequate evidence in humans. On the basis of available data, carbon tetrachloride can be considered to be a non-genotoxic compound. It is likely that the carcinogenicity of carbon tetrachloride is secondary to its hepatotoxic effects (IPCS 1999).

Bis(chloromethyl)ether (BCME) occurs as a colourless liquid and has a suffocating odour. In the past, BCME was used for chloromethylation and as an alkylating agent in the manufacture of polymers (ATSDR 1989, USEPA 1999). However, it is no longer used commercially in the United States; thus the probability of human exposure is low (ATSDR 1989). USEPA has classified BCME as a Group A, known human carcinogen (USEPA 1999). Animal studies indicate that BCME is a potent carcinogen with a short latency period. A high incidence of respiratory tract tumours has been reported in rats and pulmonary adenomas in mice exposed by inhalation. In dermally exposed mice, skin papillomas and carcinomas were observed (ATSDR 1989, USEPA 1999). Several occupational studies have reported increased incidences of lung cancer among exposed workers (ATSDR 1989, USEPA 1999).

2.3.2.4 Limitations of GC-MS

In this Chapter, proof of concept studies were conducted in order to justify the oxidising effect of NaOCl against mineralised human dentine powder and to study the probability of VOC and DBP formation. The application of ATR-FTIR has been implemented in previous studies, which has been considered as appropriate methodology to study the effects of NaOCl concentration and exposure time on the organic content of dentine. We further evaluated the effect of irrigant volume and alteration in dentine mass, when NaOCl was used at different concentrations from 1% to 5.25% v/v using GC-MS. The application of GC-EI-MS enabled the chemical analysis of chlorinated aliquots resulting from the interaction of NaOCl with dentine powder at different time intervals.

GC-MS is a powerful method for the identification of compounds; however, it is time consuming and often requires calibration with the use of gaseous or volatile samples of known pre-determined concentrations. The use of extraction solvents was avoided in order to prevent alterations in the content of the tested samples, so we opted for immediate sample injection. Due to the nature of the reactants, we came across several technical issues that hindered the experimental procedures. These issues were related to the high concentration of the chlorinated samples and the formation of salts, which could potentially block the quadrupole and the connector tubes. In addition, GC-MS proved time-consuming as for each experimental run fresh reference standards for the two components had to be prepared and the instrument had to be calibrated for each trace gas to achieve meaningful quantification. Obviously, this was not real-time monitoring and the fragmentation patterns of each component of the gas mixture must be known when electron ionization is used, as is generally the case for GC-MS.

2.4 Conclusions

The null hypothesis that the use of different NaOCl concentrations (1%, 2% and 5.25%) has no effects against the subsurface organic and inorganic degradation within mineralised dentine, associated with dentine weight variations or irrigant volume variations was rejected. Increased dentine powder weight and reduced volume of irrigant had an inhibitory effect on collagen depletion when NaOCl 1% and 2% concentrations were applied. NaOCl 5.25% exhibited the strongest effects on dentine collagen depletion regardless of dentine powder mass and irrigant

volume. In addition, NaOCl 5.25% presented moderate effects against the inorganic content of dentine.

The chemical interaction of human dentine with NaOCl 2.5% and 5% v/v resulted in the formation of toxic DBPs at all periods of observation. DBPs may also pre-exist as precursors within blank NaOCl solutions. NaOCl concentration and time did not have any effect on the degree of DBPs formation. The results of this study indicate that additional chemical parameters should be encountered when NaOCl is used as main root canal irrigant. However, the outcomes cannot be directly extrapolated in clinical conditions. The risks and drawbacks from the use of NaOCl in endodontic procedures require further analysis and critical appraisal with additional environmental and human exposure criteria. Further studies are required for the examination of the cumulative effects on dental staff and the degree of patient exposure through inhalation of the volatile phase of DBPs during endodontic treatment procedures, under conditions of good practice.

Chapter 3

Quantification of volatile compounds produced by the action of NaOCl in a model system of infected root canal content using selected ion flow tube-mass spectrometry (SIFT-MS)

3.1 Introduction

The interaction of dentine with NaOCl was further investigated to enable quantification of volatile species generated. To provide a real-time analytical method that supplements GC-MS and the other analytical methods, a more rapid and direct method of analysis of volatile organochlorine compounds was attempted using selected ion flow tube mass spectrometry (SIFT-MS).

The SIFT technique was conceived and developed almost 30 years ago (Adams & Smith 1976), and it quickly became a standard method for the study of ion–neutral reactions at thermal interaction energies (Smith & Adams 1987). This is a quantitative mass spectrometric method that exploits chemical ionization using judiciously-selected precursor positive ions that react during a defined (short) reaction time with the trace gases in air and breathe samples that are introduced into a helium stream in a flow tube. This powerful combination of the fast flow tube technique, chemical ionization, and quantitative mass spectrometry allows accurate quantitative analyses of several trace gases simultaneously in air and exhaled breath down to the parts per billion (ppb) regime of concentration on time scales of seconds. The unique advantage of SIFT-MS lies in the immediacy of the results and in absolute quantification of a wide variety of low molecular weight compounds like ammonia and some aldehydes that are not so easily analysed by GC-MS (Phillips & Greenberg 1992, Sanchez & Sacks 2003).The instrumental facility offers the option to choose precursor ions for the analysis of particular media, illustrating the versatility of SIFT-MS as a major advantage over most other analytical methods.

The SIFT-MS technique has been extensively described in literature (Smith & Španěl 2005) and a schematic illustration is presented in Figure 3-1. A mixture of precursor (reagent) ions,

including hydronium (H_3O^+), nitrosonium ion (NO^+) and dioxygenyl ion (O_2^+) are generated in a microwave discharge (Figure 3-1). Each of the mentioned reagent ions can be selected by a quadrupole mass filter and injected into a fast-flowing helium carrier gas in a flow tube (Figure 3-

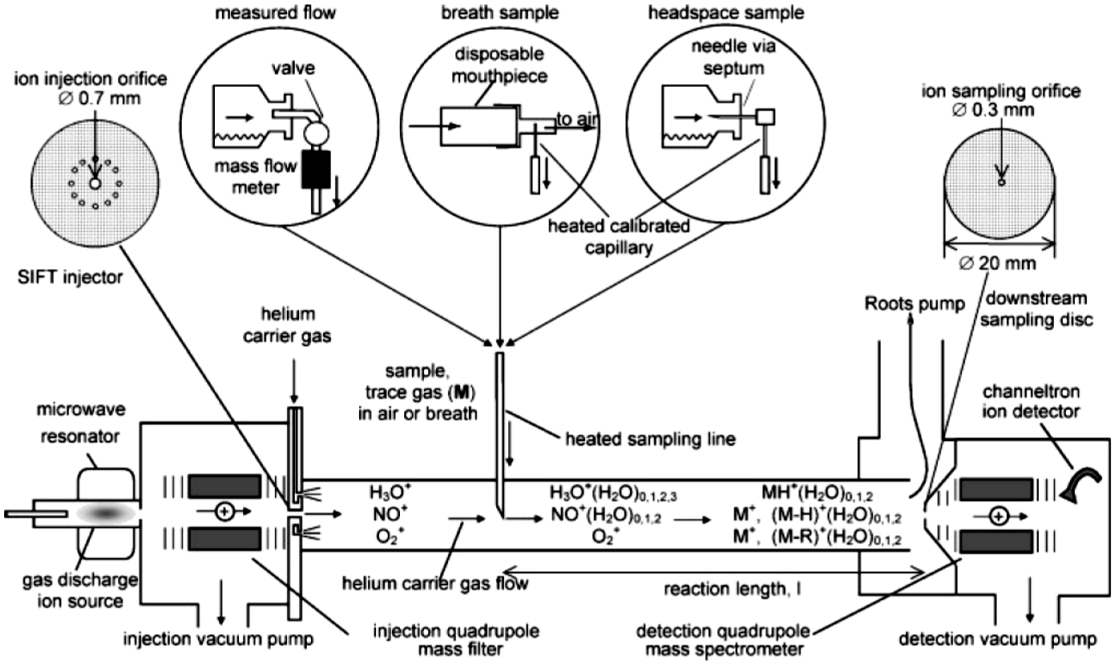


Figure 3-1 The SIFT-MS instrument scheme and the relevant sampling methods *(adopted by Smith & Spaněl 2005)*

-1). The sample gas to be analysed naturally flows into the helium at a controlled rate via a calibrated capillary by virtue of the atmospheric pressure of the sample gas and the much lower pressure of the helium (typically 1mbar). The chosen reagent ion then reacts with the trace components in the sample (to the exclusion of the major air components) to generate product (analyte) ions. The reagent ions and analyte ions are mass analysed by a quadrupole mass spectrometer/ion counting system (Figure 3-1). Thus, the characteristic analyte ions identify the neutral trace components present in the sample and the count rates provide their concentrations in real time.

The reaction of H_3O^+ with the large majority of organic compounds is seen to proceed via exothermic proton transfer (Smith & Španěl 2005). The nascent product ion of the proton transfer reaction of H_3O^+ with molecule M is MH^+, which is often observed as the only stable product ion. However, these MH^+ nascent ions sometimes dissociate to form ions like $(M\text{-}OH)^+$ and $(M\text{-}OR)^+$,

with R being a radical such as methyl group (CH_3) and ethyl group (C_2H_5). The formation of $(M\text{-}OH)^+$ ions results from the elimination of H_2O molecules from the nascent MH^+ ions.

The reactions of NO^+ with organic molecules are more varied than are those of H_3O^+, but fortunately they usually result in only one or two product ions (Smith & Španěl 2005). Several major reaction processes are represented in NO^+ reactions with organic molecules M, including charge transfer producing M^+ ions, hydride ion (H^+) transfer producing $(M\text{-}H)^+$ ions, hydroxide ion (OH^+) transfer producing $(M\text{-}OH)^+$ ions, alkoxide ion (OR^-) transfer producing $(M\text{-}OR)^+$ ions, and ion–molecule association producing NO^+M ions.

The ionisation energy of O_2 molecules (12.06eV) is appreciably greater than most organic molecules (Lias *et al.* 1988), which ensures that their reactions with O_2^+ ions mostly proceed either via simple non-dissociative charge transfer producing the parent radical cation M^+ or via dissociative charge transfer reactions resulting in two or sometimes several fragment ions (Anicich 2003). O_2^+ precursor ions are most valuable for the detection and quantification of small molecules that do not react with either H_3O^+ or NO^+ ions. O_2^+ also reacts rapidly with ammonia producing only NH_3^+ and this acts as a valuable check on H_3O^+ quantification of ammonia, in which the product ion is NH_4^+ (Španěl *et al.* 1998).

SIFT-MS instruments can be operated in two modes, the full-scan (FS) mode and the multiple-ion monitoring (MIM) mode (Smith & Španěl 2005). In FS mode, a complete mass spectrum is obtained by sweeping the detection quadrupole ion over a selected m/z ratio range for a chosen time whilst a sample is introduced into the carrier gas at a steady flow rate. The count rates of the ions are then calculated from the numbers of counts and the total sampling time for each ion. The count rates are stored, and then displayed by the on-line computer on a linear or semi-logarithmic scale. The mass spectra are interpreted by relating the product ion peaks to the trace gases present in the sample. In MIM mode, the instrument monitors the count rates of the precursor ions and those of selected product ions. This is achieved by rapidly switching the downstream mass spectrometer between the masses of all the primary ions and the selected product ions and dwelling on each of these masses for a predetermined short time interval. This real-time monitoring is possible because of the fast time response of SIFT-MS, largely determined by the fast flow rates of the carrier gas along the flow tube and the sample gas along the inlet

tube. A specially developed interface driver computer program performs the switching between different ions and the acquisition of the count rates during the dwell intervals (Španěl 2003).

SIFT-MS is a valuable addition to the available trace gas analytical techniques. It has applications in many research and clinical domains including environmental and agriculture sciences, health and safety practice, medical and veterinary sciences, and in the food industry. Table 3-1 summarises the various areas of SIFT-MS applications.

Table 3-1 Areas of application of SIFT-MS.

Domains	**SIFT-MS application**	***References***
Environmental Science	• Laboratory air • Biological monitoring • Polluted town air • Exhaust gases • Monitoring of cargo containers	*Španěl et al. 1996* *Smith et al. 1998* *Smith et al. 2004* *Hurst et al. 2005* *Baur et al. 2006*
Biological Science	• Emissions from Porcine Feces and Urine • Rumen Gases • Soil Science	*Dewhurst et al. 2001* *Milligan et al. 2002* *Clough et al. 2003*
Food Science	• Quantification of aroma compounds in fermentation • Oil quality • Food flavour analyses • Volatile compounds emitted by fruits and vegetables • Volatile organic compounds related to sensory quality	*Davis et al. 2005* *Olivares et al. 2010* *Ozcan & Barringer 2011* *Agila & Barringer 2013* *Dryahina et al. 2018*
Human Physiology Healthy State	• Common Breath Metabolites • Influence of diet on breath volatiles • Ethanol metabolism • Isoprene in Breath • Oral microflora and difference between nasal and oral exhalations • Exercise • Urine Ketones in urine • Volatile markers of ovulation in urine • Skin Release of volatile compounds by skin	*Senthilmohan et al. 2000* *Diskin et al. 2003* *Smith et al. 2007* *Pysanenko et al. 2008* *Turner et al. 2008* *Enderby et al. 2009* *Smith et al. 2010*

Exposure of people to volatile compounds	• Smoking and Passive Smoking • Substance abuse • Exposure to Volatile Solvents	*Senthilmohan et al. 2001* *Smith et al. 2002* *Wilson et al. 2002* *Bloor et al. 2008*
Clinical Science Diseased State	• Urinary infection	*Storer et al. 2011*
	• Cancer	*Spanel et al. 1999* *Smith et al. 2003* *Spaněl & Smith 2008* *Sulé-Suso et al. 2009* *Huang et al. 2013* *Kumar et al. 2013*
	• Microbial infection	*Carroll et al. 2005* *Allardyce et al. 2006* *Mariam et al. 2017* *Slade et al. 2017*
	• Renal failure - Breath biomarkers of kidney dysfunction and total body water monitoring	*John et L. 2010* *Wang et al. 2016* *Demirjian et al. 2017*
	• Helicobacter pylori Infection	*Smith & Spanel 1996* *Vaira et al. 2002*
	• Cystic fibrosis	*Gilchrist et al. 2013* *Smith et al. 2016* *Španěl et al. 2016*
	• Diabetes	*Turner et al. 2009* *Smith et al. 2011* *Storer et al. 2011*
	• Halitosis	*Pysanenko et al. 2008* *Ross et al. 2009*
Security	• Detection of volatile markers of explosives • Fumes of explosions	*Wilson et al. 2006* *Sovová et al. 2010* *Civis et al. 2011*

The results of the proof of concept study (Chapter 2) showed that the emergence of toxic chlorinated DBPs and VOCs from the interaction of NaOCl with human mineralised dentine

powder requires further examination due to the potential hazardous drawbacks during root canal irrigation. SIFT-MS is a rapid, direct and sensitive analytical tool for the quantification of VOCs and DBPs compared to GC-MS (Smith & Španěl 2005, Španěl & Smith 2011). The use of SIFT-MS may prove to be a valuable method to study the potential reactions of NaOCl with organic material in a laboratory model of infected tooth biomaterial.

The aim of this study was to screen and quantify ex vivo, the formation of chlorinated DBPs and VOCs resulting from the short-term interaction of NaOCl 2.5% v/v with human dentine, endodontic pathogens, serum albumin and their combinations, using SIFT-MS and determine the suitability for this analysis. The null hypothesis was that the interaction of NaOCl with these components of infected root canal does not result in the formation of chlorinated DBPs and VOCs.

3.2 Materials and methods

3.2.1 Dentine preparation

Fifteen freshly extracted intact and fully developed human impacted and semi-impacted mandibular third molars that were free of cracks, fractures, caries, abrasions and discolouration were collected. All procedures were approved and conducted in accordance with the protocol outlined by the Research Ethical Committee (Wales REC 4, 14/WA/1004, UK). Access cavities were performed to remove pulpal debris with stainless steel hand files and distilled water. The enamel and cementum layers were removed with high speed diamond conical burs (Ash HiDi Friction Grip Taper 554-Medium Grit, Dentsply, Weybridge, UK). The remaining dentine bulk was then reduced to a powder state by using low speed round burs (Steel Round Finishing Bur size E, Dentsply, Weybridge, UK).

Dentine powder particle size distribution was measured with a laser diffraction particle-size analyser (CILAS 1180, Orleans, France), which could analyse particles within the range 0.04-2500 µm. Five samples of dentine powder (10mg) were randomly selected, inserted into a water tank and ultra-sonicated for 30s. Particle size distribution was expressed by the obtained values from the cumulative distributions with measures of central tendency [median diameter (d50)] and distribution width (relative span=d90-d10/d50) (Jillavenkatesa *et al.* 2001). D50 values varied from

17.66μm to 31.03μm and relative span values varied from 1.10 to 2.62. The total dentine powder sample was equally divided into experimental and control groups, each one containing 50mg, sterilised in an autoclave and kept at -80 ^{0}C until further use, to prevent degradation of collagen components.

3.2.2 Characterisation of root canal irrigant NaOCl 2.5% v/v

The available chlorine content of a 10-15% v/v stock solution of NaOCl (Sigma Aldrich, Gillingham, UK) was verified with a standard iodine/thiosulfate method (Vogel 1962). The chemical reactants used were: (i) Sodium thiosulphate solution (0.2M) which was prepared from 50 g sodium thiosulphate pentahydrate crystals (99.8% purity, Alfa Aesar, Heysham, UK) dissolved in 1 L HPLC water (Chromasolv, Sigma Aldrich, Gillingham, UK), and 25 mg of sodium carbonate (VWR, UK) were added to adjust the pH of the solution to alkaline levels, (ii) potassium iodate (0.05M) (Sigma Aldrich, Gillingham, UK) and potassium iodide (0.24M) (Alfa Aesar, Heysham, UK), (iii) sulphuric acid (3M) (Fisher Scientific, Loughborough, UK) and (iv) potato starch (2% w/v) (VWR, Lutterworth, UK).

Titrations (n=3) of sodium thiosulphate consumption were carried out after the standardisation of the titrating agent with potassium iodate (0.05M) as a primary standard at 0.22M. The concentration of NaOCl stock solution was 1.474M. Stock NaOCl solution was diluted with HPLC water to obtain 2.5% v/v NaOCl (0.34M). The NaOCl solutions were stored in air-tight dark containers at 5^{o} C until further use.

3.2.3 Development of planktonic multi-microbial colonies

Propionibacterium acnes, *Staphylococcus epidermidis*, *Actinomyces radicidentis* and *Streptococcus mitis,* recovered in previous study as predominant taxa from the root canals of teeth with refractory endodontic infections, were selected (Niazi *et al.* 2010). *Enterococcus faecalis* strain OMGS 3202 was also included (Dáhlen *et al.* 2000). To establish the microbial growth, the strains were cultured anaerobically at 37 °C for one week on Fastidious Anaerobe

Agar (FAA, Lab M, Heywood, UK) supplemented with 5% defibrinated horse blood. Individual starter cultures of each species were collected with inoculation loops (Cole-Palmer, UK), added to phosphate buffered saline (PBS) and incubated anaerobically at 37°C for 3h in anaerobic workstation (MACS-MG-1000, Don Whitley Scientific Ltd, UK). The absorbance was adjusted with PBS to 0.5 at 540nm to obtain 10^7 cells ml^{-1} (Labsystems iEMS Reader MF, Basingstoke, UK).

3.2.4 Interaction of NaOCl in ultrapure water with different sources of NOM

To study the interaction of NaOCl with combined sources of NOM and their effect on the formation of any chlorinated DBPs and other VOCs, four experimental groups containing aqueous suspensions of NaOCl 2.5% v/v and one control group containing ultrapure water and no NaOCl were developed (Table 3-2). The total volume for each group was 10ml, equally divided into four aliquots (2.5ml), according to the type and combination of NOM sources (Table 3-2). Dentine powder (50mg) was suspended in 2.5ml ultrapure water (Sigma Aldrich, UK). Multi-microbial liquid suspensions of 2.5ml volume were acquired after the addition of 0.5ml aliquots of each of the five planktonic microbial species. Bovine serum albumin (BSA) solution (4% w/v) was obtained following the addition of BSA powder (A1933, Sigma Aldrich, UK) into ultrapure water (Sigma Aldrich, UK). All samples were injected into 20ml universal tubes and stirred at 37^0C under both aerobic and anaerobic conditions for 30min to approximate a clinically realistic time of root canal irrigation procedures and ensure homogeneous contact between the reactants. After this period, all samples were centrifuged (2min at 200rpm) and the supernatants were collected and stored at -80^0C until analysis. The preparation of the aliquots and their associated samples as well as the experimental procedures were conducted in triplicates.

Table 3-2 Group allocation and interaction of different sources of NOM with 2.5% NaOCl and 50mg of powdered dentine (Groups 1-4) and Ultrapure water (Control group). Each sample was studied under aerobic and anaerobic conditions.

TOTAL VOLUME	**EXPERIMENTAL GROUPS**				**CONTROL GROUP**
10 mL	**Group 1**	**Group 2**	**Group 3**	**Group 4**	
2.5 mL	NaOCl	NaOCl	NaOCl	NaOCl	Ultrapure water
2.5 mL	Dentine powder	Planktonic multi-microbial suspensions	Dentine powder	Dentine powder	Dentine powder
2.5 mL	Ultrapure water	Ultrapure water	Planktonic multi-microbial suspensions	Planktonic multi-microbial suspensions	Planktonic multi-microbial suspensions
2.5 mL	Ultrapure water	Ultrapure water	Ultrapure water	Bovine serum albumin 4%	Bovine serum albumin 4%

3.2.5 SIFT-MS analysis

For analysis, the samples were defrosted in air. Analysis of the headspace volatile compounds was carried out in real-time by SIFT-MS. Prior to analysis, three replicate 2.5ml aliquots of each sample were placed into a sample bag constructed from 50cm length, 65mm diameter Nalophan NA (Kalle, UK), which was then filled with purified air and sealed prior to incubation at 37^0C. After equilibrium between the liquid and headspace above it (30min), the headspace was sampled directly into the SIFT-MS via a heated, calibrated capillary that defines the headspace sample flow rate, as is necessary for absolute quantification of VOCs. The analytical downstream quadrupole mass spectrometer was scanned over the range of mass-to-charge ratio, m/z, using the three reagent ions H_3O^+, NO^+ and O_2^+ independently. From the m/z values of the analyte ions and their count rates, and using the kinetics database stored in the instrument library, the concentrations of the identified VOCs were immediately obtained (Smith & Španěl 2005, Španěl & Smith 2011).

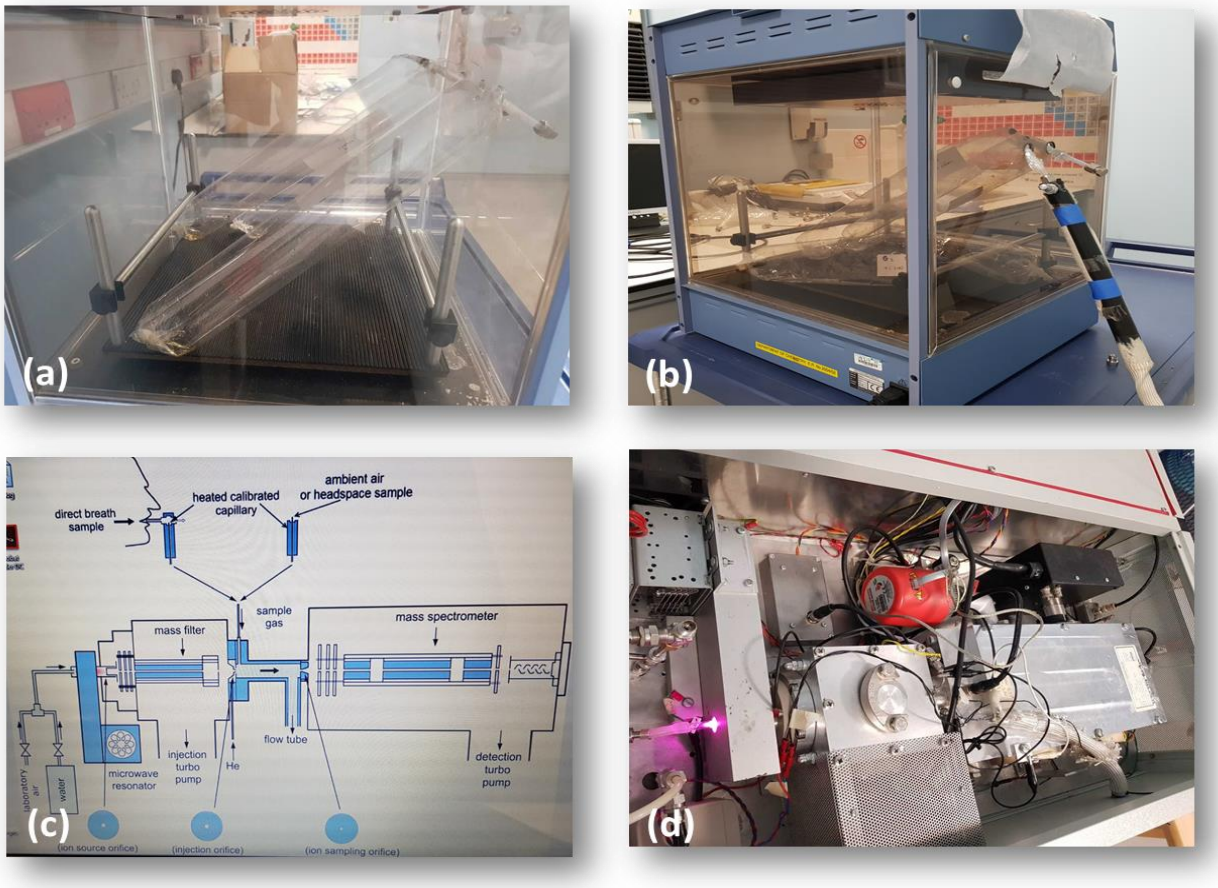


Figure 3-2 Stages of SIFT-MS analysis (a) Sample placement into Nalophan bag and incubation. (b) Application of heated calibrated capillary to sample headspace directly into the SIFT-MS. (c) Selection of reagent ions via computer software. (d) Activation of scanning process in mass spectrometer.

3.3 Results

3.3.1 Compounds common to biological media

The headspace of all samples contained several common compounds that are often seen in biological media headspace (Table 3-3) (Turner *et al.* 2008). Sample spectra using the H_3O^+ reagent ion for the Control Group and experimental Group 4 are presented in Figures 3-3 & 3-4, where analyte ions derived from ammonia (NH_3), acetaldehyde (CH_3CHO), acetone (CH_3COCH_3), methanol (CH_3OH), ethanol (C_2H_5OH) can be seen.

The characteristic ions of ammonia were also detected in the O_2^+ reagent ion spectra (Fig 3-4). No significant differences were observed between samples in aerobic and anaerobic

conditions, with the exception of acetone, that presented higher concentrations in anaerobic conditions for Groups 1, 2 and 3. The reason for this is not clear. With regard to ammonia, Group 4 was significantly different from all the other groups. The ammonia concentrations present in the headspace of Group 4 samples was several times greater than in the other groups' headspace, although it was present in all samples, in aerobic and anaerobic conditions, including the control group.

3.3.2 Nitrogen-containing organic compounds; acetonitrile (CH_3CN)

Evidence of the presence of amines or amides in the H_3O^+ spectrum (Figure 3-4) was also noticeable. These two compounds were not seen in the control group, but in experimental groups 1-4, aerobically and anaerobically, with the largest signals in groups 2 and 3 (although not substantially larger than groups 1 and 4). The characteristic even numbered ions when using H_3O^+ reagent ions usually indicate nitrogen-containing compounds. Thus, the analyte ion at m/z 88 (and its monohydrate at m/z 106) is most probably either $C_5H_{13}N$ (pentylamine) or C_4H_9NO (butyramide). The minor peak at m/z 102 (plus its monohydrate m/z 120) is most probably either $C_6H_{15}N$ (dipropylamine) or $C_5H_{11}NO$ (pentanamide).

However, there is no doubting the identity of the much more pronounced characteristic analyte ions of acetonitrile (CH_3CN) in the headspace of all groups containing NaOCl (not present in the to the control group headspace). The reaction of acetonitrile with H_3O^+ reagent ions in humid media almost uniquely produces four analyte ions $CH_3CNH^+(H_2O)_{0,1,2,3}$ with m/z values of 42, 60, 78, 96, which are clearly seen in Figure 3-4 (Španěl & Smith 1998, Abbott *et al.* 2003). It is present in easily quantifiable concentrations by SIFT-MS (Table 3-3).

Table 3-3 Mean concentrations and range [minimum-maximum] of 2 or 3 samples (mg L^{-1} by volume) from the analyses by SIFT-MS using H_3O+ and NO+ reagent ions of some compounds present in the headspace of control group and experimental groups 1-4 in aerobic and anaerobic conditions.

Sample	Ammonia Mean [range]	Acetaldehyde Mean [range]	Acetone Mean [range]	Methanol Mean [range]	Ethanol Mean [range]	Acetic acid Mean [range]	Acetonitrile Mean [range]
Control air	0.654 [0.479-0.869]	0.056 [0.048-0.065]	0.016 [0.014-0.017]	0.089 [n/d-0.178]	0.114 [0.078-0.150]	0.074 [0.040-0.117]	0.009 [n/d-0.017]
Control no air	0.566 [0.325-0.738]	0.006 [n/d-0.013]	0.069 [0.009-0.128]	0.050 [n/d-0.100]	0.368 [0.103-0.634]	0.098 [0.026-0.210]	0.004 [n/d-0.009]
Group 1 air	0.176 [0.153-0.211]	0.130 [0.128-0.132]	0.038 [0.032-0.043]	0.036 [n/d-0.071]	0.057 [0.006-0.108]	0.119 [0.066-0.149]	0.162 [0.156-0.167]
Group 1 no air	0.234 [0.098-0.497]	0.125 [0.118-0.132]	0.393 [0.294-0.492]	0.019 [n/d-0.039]	0.014 [0.006-0.021]	0.057 [0.035-0.092]	0.299 [0.262-0.335]
Group 2 air	0.409 [0.061-0.936]	0.048 [0.039-0.057]	0.052 [0.040-0.064]	0.091*	0.011 [n/d-0.021]	0.043 [0.030-0.056]	0.174 [0.127-0.221]
Group 2 no air	0.236 [0.180-0.339]	0.045 [n/d-0.089]	0.189 [0.160-0.219]	n/d [n/d-n/d]	0.003 [n/d-0.006]	0.058 [0.014-0.107]	0.272 [0.183-0.359]
Group 3 air	0.152 [0.091-0.192]	0.128 [0.079-0.177]	0.183 [0.167-0.199]	0.045 [n/d-0.090]	0.051 [0.050-0.052]	0.060 [0.053-0.073]	0.400 [0.260-0.540]
Group 3 no air	0.284 [0.120-0.590]	0.048 [0.019-0.076]	0.693 [0.577-0.807]	0.161 [0.040-0.281]	0.041 [0.013-0.069]	0.131 [0.034-0.260]	0.653 [0.581-0.725]
Group 4 air	5.563 [2.270-10.663]	0.825 [0.580-1.070]	0.046 [0.045-0.048]	0.100 [0.069-0.131]	0.093 [0.091-0.095]	0.053 [0.012-0.079]	0.316 [0.226-0.407]
Group 4 no air	6.664 [3.951-10.250]	1.023 [0.815-1.230]	0.046 [0.036-0.057]	0.214 [n/d-0.429]	0.130 [0.111-0.148]	0.102 [0.003-0.227]	0.200 [0.098-0.302]

*Only one data point

n/d= non detectable.

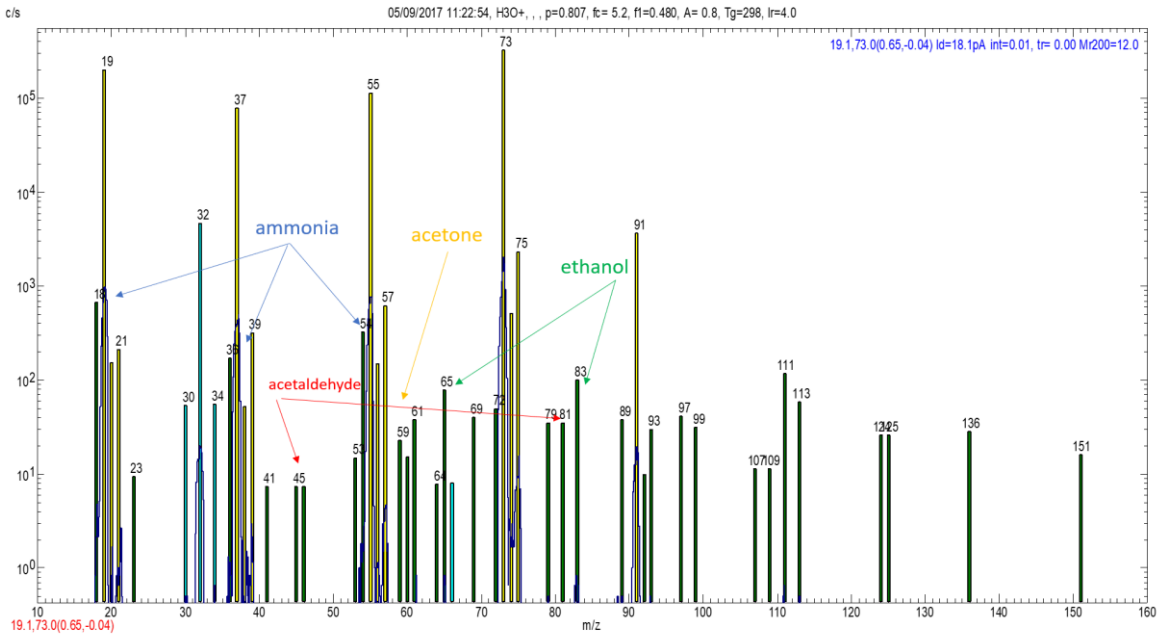


Figure 3-3 H_3O^+ spectrum of control group sample in air. Ions indicating ammonia, acetone, acetaldehyde and ethanol are shown on the spectrum.

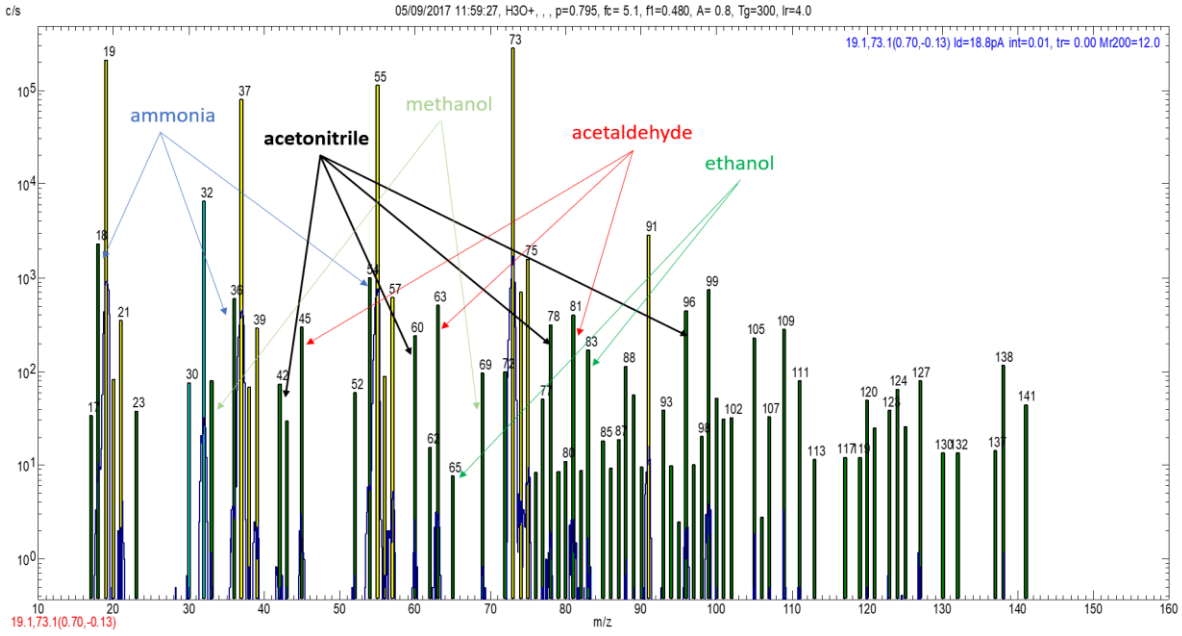


Figure 3-4 H_3O^+ spectrum of group 4 sample in air. Ions indicating the presence of acetonitrile (42, 60, 78, 96) are indicated. Ammonia, acetaldehyde, ethanol and methanol are also indicated.

3.3.3 Production of chloroform ($CHCl_3$)

In view of the previous work that reported the production of organochlorine DBPs when using NaOCl for root canal irrigation (Varise *et al.* 2014), the SIFT-MS spectra were carefully inspected for the presence of chlorine-containing analyte ions. Unfortunately, chlorinated organic compounds react only slowly with H_3O^+ and NO^+ and so these reagent ions are not very useful for the analysis of these compounds. However, they usually react rapidly with O_2^+ ions (Španěl & Smith 1999). Ions containing one chlorine atom can often be recognised on mass spectra because the peak heights (intensities) of the two isotopologues (separated by two m/z units) will be in the ratio of the naturally occurring abundance ratio of ^{35}Cl to ^{37}Cl isotopes which is statistically 3:1 (75%:25% peak intensities). Hence, by recognising analyte ions that differ by 2 m/z units that have peak intensity ratios of 3:1, it is possible to identify monochlorinated ions and suggest molecular formulae.

For ions containing two chlorine atoms, three isotopologue ions will be formed separated by 2 m/z units with the peak height intensities in the ratio 9:6:1 (56%:38%:6%). Even though the spectra obtained using O_2^+ reagent ions are very "busy", because these energetic reagent ions can fragment analyte neutral molecules and produce multiple mass spectral peaks, as can be seen in Figure 3-5, analyte ions at m/z values of 83, 85 and 87, with peak intensities close to the statistical ratio expected for an ion containing 2 chlorine atoms, are present in all the spectra obtained for the analysis of the samples containing NaOCl, but not in the control sample spectra. The only candidate for this dichlorinated ion is $CHCl_2^+$. This closed shell ion is known to be the product of the O_2^+ reaction with chloroform ($CHCl_3$) (Španěl & Smith 1999). Thus, chloroform is present in the headspace of all the samples containing NaOCl at easily measured concentrations that exceed that of ammonia in Groups 1-4 (Table 3-4). In the experimental Groups 1-4, the concentrations presented an increasing tendency from 1ppm to 3.4ppm, as the variable sources of NOM reactants became more concentrated.

To determine whether these findings arose through the reaction of the NaOCl with the organic content (dentine, bacteria and serum) and not the material of the Nalophan sample bag, NaOCl was added to the sample bag to which hydrocarbon free air was added. The bag was incubated for 30min at 37^{o}C and then analysed by SIFT-MS. No evidence of chloroform,

acetonitrile or any of the other product ions mentioned here were detected, so the findings shown above are not down to reactions of NaOCl with the bag material.

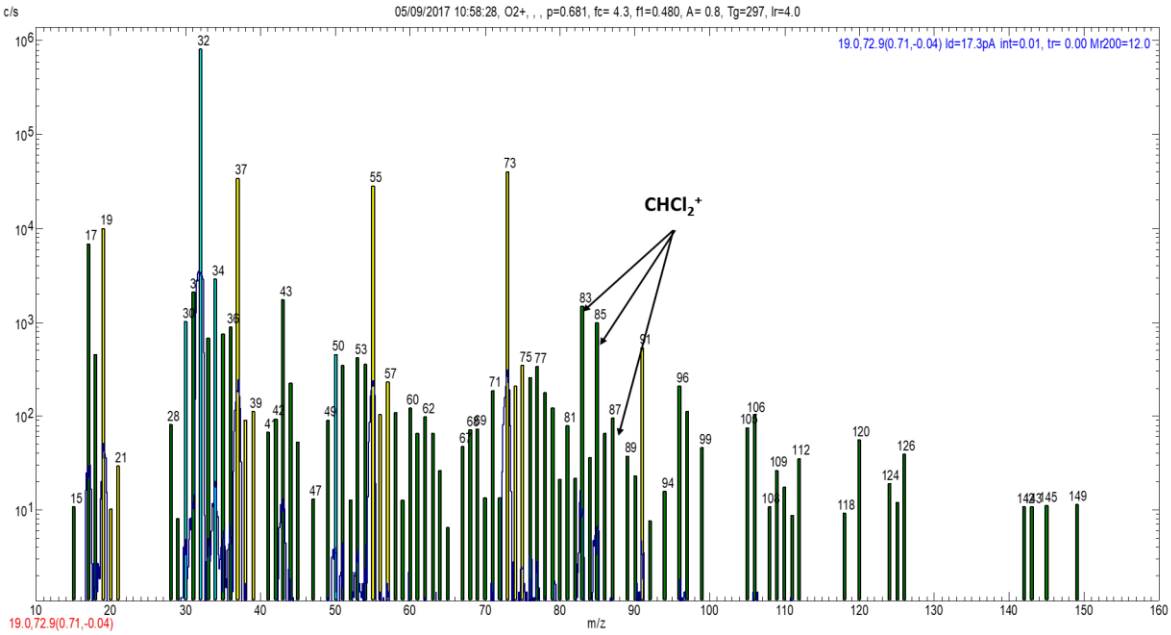


Figure 3-5 O_2^+ spectrum from group 4 in anaerobic conditions showing ions at 83, 85 & 87, and their respective ion counts (83[1485], 85[1342], 87[97]), representing $CHCl_2^+$ derived from the O_2^+ reaction with chloroform.

Table 3-4 Ion intensities of ions (count rates) as relative percentages (%) in parentheses at m/z 83, 85 and 87 using O_2^+ reagent ions and thus the concentration of chloroform (mg L^{-1} by volume). Note that the mean peak intensity percentages (%) are precisely in line with the statistical predictions (given in the text).

Sample	**m/z 83**	**m/z 85**	**m/z 87**	**$CHCl_3$ (mg L^{-1})**
Control air	n/d	n/d	n/d	n/d
Control no air	n/d	n/d	n/d	n/d
Group 1 air	931 (61)	487 (32)	112 (7)	1.826
Group 1 no air	505 (54)	332 (37)	95 (10)	1.038
Group 2 air	445 (52)	333 (39)	80 (9)	1.061
Group 2 no air	655 (59)	398 (36)	60 (5)	1.292
Group 3 air	1092 (48)	935 (41)	245 (11)	2.767
Group 3 no air	1372 (59)	783 (34)	150 (6)	2.072
Group 4 air	1644 (52)	1342 (43)	152 (5)	3.474
Group 4 no air	1485 (58)	983 (38)	97 (4)	2.622
Mean percentages (%)	(56)	(38)	(6)	

n/d: non detectable.

3.4 Discussion

In this study, a novel analytical methodology was proposed and validated for the detection of VOCs and DBS following the interaction on NaOCl with components of an infected root canal system, as pontential osurces of NOM. SIFT-MS analysis proved to be an effective method and the real-time trace analysis of volatile compounds revealed the formation of chloroform and acetonitrile. In addition, several compounds present in biological media such as ammonia, acetaldehyde, acetone, methanol, ethanol and acetic acid were detected and quantified (Turner *et al.* 2008). The technique is rapid, direct and quantitative and is ideally suited for the study of this model system.

Many root canal disinfectants including NaOCl present excellent antimicrobial activity *in vitro* whereas *in vivo* they often fail to completely kill all microbes. This might be related to the presence of dentine, organic and inorganic matter which can interact with NaOCl, thereby reducing its efficacy (Moorer & Wesselink 1982, Macedo *et al.* 2010). An aqueous model system of existing components within infected root canals was used to examine the formation of organochlorines. The use of dentine powder, multi-bacterial planktonic strains and bovine serum albumin has been implemented in previous studies to test their inhibitory action against NaOCl antimicrobial capacity. Dentine powder was shown to have an inhibitory effect on the antimicrobial activity of 1% NaOCl against *E. faecalis*, which was dependent on the concentration as well as on time of incubation with dentine powder before addition of bacteria (Haapasalo *et al.* 2000). Sassone *et al.* (2003) investigated the inhibitory effect of bovine serum albumin (BSA) 0.5% on the antimicrobial activity of 1% and 5% NaOCl with no inhibition. A higher BSA concentration (6.7%) also reported an inhibitory effect against NaOCl 0.08-6% on the microbial species tested (Pappen *et al.* 2010). However, the fate of available chlorine consumption or the nature of the chemical reactions involved was not discussed in any of the previous studies. In this study, the sole interaction of NaOCl with BSA was not investigated as an independent experimental group, as it does not offer any clinical relevance. In clinical conditions and within an infected root canal, NaOCl definitely interacts with dentine, dispersed planktonic bacteria and their combination, but cannot not interact solely with serum albumin. Serum albumin can flow in the root canal through the root apex, deriving from apical inflammatory fluid exudation. However, from the results it is

clearly shown that the presence of BSA as an additional source of NOM contributed to the enhanced formation of ammonia and chloroform in Group 4, compared to Groups 1-3.

To date, our knowledge about the chemical reactions of NaOCl in contact with organic biomaterials is summarised to the series of chemical reactions, which were previously described in Chapter 1. Those include the reaction of sodium hydroxide (NaOH) with fatty acids and amino acids leading to the formation of soap and glycerol as well as salt and water, respectively (Estrela *et al.* 1995). The aqueous phase of HOCl reacts with amino acids leading to chloramine (NH_2Cl) formation. Further reaction of NH_2Cl with HOCl and OCl- leads to hydrolysis and degradation of amino acids, formation of volatile dichloramines (Cl_2HN) and trichloramines (NCl_3). Chloramines are able to interfere with cellular metabolism leading to irreversible oxidation of SH- (sulphydryl groups) of essential bacterial enzymes (How *et al.* 2017).

The experimental procedures were conducted under both aerobic and anaerobic conditions to justify any alterations in the formation of VOCs and DBPs. From an ecologic perspective, the conditions within infected root canal systems vary at different depths. The coronal third presents high oxygen tension, availability of nutrients from oral cavity and microorganisms can be directly exposed to antimicrobial agents (Chavez de Paz 2007). Oxygen tension and nutrients present a declining tendency from the middle to apical root third. However periapical tissues may offer different sources of nutrition in the apical third and microorganisms are less vulnerable to antimicrobial agents due to anatomical restrictions (Chavez de Paz 2007). After access cavity and during root canal preparation and irrigation, oxygen may progressively diffuse, however the least approachable areas in the apical root third may still be subjected to treatment under oxygen-low or anaerobic conditions (Chavez de Paz 2007). To-date, we have no available data regarding the possible selectivity of NaOCl oxidation capacity under aerobic or anaerobic conditions. The results of this study showed that no significant differences were observed between samples in aerobic and anaerobic conditions, with the exception of acetone, that presented higher concentrations in anaerobic conditions for groups 1, 2 and 3. The reason for this is not clear.

The presence of ammonia was evident in both control and experimental groups. Previous studies of the reactions of H_3O^+ with several amines show that these organic species are very efficiently protonated by H_3O^+ by virtue of the high probability of activity of amines. In some of the primary amine reactions, NH_3 molecules are eliminated from the nascent MH^+ ions resulting in

$(M\text{-}NH2)^+$ hydrocarbon ions, a phenomenon which does not occur in the secondary and tertiary amine reactions, suggesting a way to distinguish between these amine isomers using SIFT-MS (Španěl & Smith 1998, 1999). O^{2+} also reacts rapidly with ammonia producing only $NH3^+$ and this acts as a valuable check on H_3O^+ quantification of ammonia, in which the product ion is NH_4^+ (Španěl & Smith 2011).

The presence of ammonia in the control group is as a result of the emission from the bacterial population, which is produced from the metabolism of peptides and amino acids, especially via L-aspartate catabolism (Bernier *et al.* 2011). Group 4 was significantly different from all the other groups. The ammonia concentrations present in the headspace of group 4 samples was several times greater than in the other experimental groups', both in aerobic and anaerobic conditions. This suggests that ammonia is efficiently produced by the reaction of the NaOCl with BSA, since this material is not present in the other samples containing NaOCl (groups 1-3). It is well known that serum albumin and hypochlorite interaction lead to protein injury and degradation (Sharonov *et al.* 1989). One possible explanation may relate to hydrolysis that protein molecules are undergoing and lead to ammonia release (Warner & Cannan 1942). Assuming that chloramine (NH_2Cl) formation is prominent due to interaction of NaOCl with higher levels of organic matter in group 4, it can be suggested that ammonia formation may also originate from the decomposition of chloramine in alkaline aqueous conditions according to the reaction: $3NH_2Cl + 3OH^- \rightarrow NH_3 + N_2 + 3Cl^- + 3\,H_2O$ (Ura & Sakata 2007). Apart from ammonia, a higher level of acetaldehyde release in group 4 than the control or groups 1-3 was observed, under anaerobic conditions. This implies that a specific reaction of NaOCl with BSA may have occurred.

An unexpected finding was the formation of acetonitrile (CH_3CN) in all groups containing NaOCl in comparison to the control group. This compound is not usually seen in biological samples but it is often seen on human breath in smokers (Abbott *et al.* 2003). Acetonitrile was readily detected by SIFT-MS in urinary headspace of smokers at levels dependent on the cigarette consumption, but is practically absent from the breath and urine headspace of non-smokers.

A major question that needs further investigation is how these volatile nitrogenous compounds are formed and whether their direct analysis by SIFT-MS in the headspace may offer

insight into the reactions of NaOCl with proteins and amino acids. One possible explanation for the formation of acetonitrile is by the reaction of NaOCl with aldehydes and monochloramines, which has previously been investigated using solid phase microextraction (SPME) and GC-MS (Kimura *et al.* 2015). Chloramines form following the degradation of proteins and oxidation of aminoacids by NaOCl (Hawkins *et al.* 2003). The existence of acetaldehyde in both control and experimental biological samples can initiate a chemical reaction leading to formation of acetonitrile. In the study of Kimura *et al.* (2015), two additional by-products were identified including N-chloroacetamide and N,2-dichloroacetamide which appear to present significant toxicity (Kimura *et al.* 2015). However, under the experimental conditions of this study they were not found but may potentially exist as reaction intermediates.

Acetonitrile is a clear colourless liquid with a sweet ethereal odour predominantly used as a solvent in the manufacture of perfumes and pharmaceuticals, in fibre, plastic, battery industries and in chemical laboratories for the detection of materials such as pesticide (USEPA 1985). No data is available on its carcinogenic effects in humans; EPA has classified it as non-carcinogenic to humans. No information is also available on the reproductive or developmental effects of acetonitrile in humans (USEPA 1987).

Despite its low carcinogenic risk, acetonitrile can be absorbed into the body by inhalation of its vapour and by dermal absorption through the skin and by ingestion in both humans and laboratory animals (USEPA IRIS 2008). Exposure to 160ppm for 4 hours causes flushing of the face and a feeling of constriction in the chest. Concentrations up to 500ppm acetonitrile through inhalation exposure causes irritation in the nose, throat and mucous membranes in humans, and higher concentrations can produce weakness, nausea, and convulsions (USEPA 1985, 1987). European Agency for Safety and Health at Work has established indicative occupational skin exposure limit values for acetonitrile at 70mg/m^3 or 40ppm per 8 working hours (EU Commission Directive 2006/15/EC).

Its release when used in root canal irrigation should offer little concern at the relatively low levels that are generated, but an occupational risk assessment has not been performed in dental premises and in particular in endodontic surgeries yet, despite the daily use of large volumes of NaOCl for endodontic treatments. In view of the present discovery, perhaps risk assessment of acetonitrile should be carried out.

As mentioned in the Introduction, Varise *et al*. (2014) reported the formation of organochlorine compounds, including chroloform, following the 15-min interaction of NaOCl with bovine dentine powder and pulp tissue fragments. In another study, the formation of trihalomethanes and other DBPs was documented using gas chromatography with electron capture detector (GC-ECD), following the 1-h interaction of chlorine (1-3mgl^{-1}) with *E. coli* and *Pseudomonas aeruginosa* bacterial cells (Wang *et al.* 2013). The combined findings of bacterial inactivation and DBP formation imply that breaking down bacterial cells provides organic precursors for DBP formation (Wang *et al.* 2013). This evidence strongly supports the pathway for DBP formation from pure bacterial biomaterials (Wang *et al.* 2013). An alternative pathway for the formation of $CHCl_3$ may derive from the interaction of NaOCl with acetone, which is present in the headspace, according to the historically known haloform reaction: $3NaOCl + C_3H_6O \rightarrow CHCl_3 + 2NaOH + NaOCOCH_3$ (Reynold *et al.* 1934).

The production of chloroform, a trihalomethane, may have implications in dentistry. It has been shown to be carcinogenic in animals after oral exposure, resulting in an increase in kidney and liver tumours (ATSDR 2017). The United States Environmental Protection Agency (USEPA) has classified chloroform as a probable human carcinogen (USEPA 1987). The World Health Organisation (WHO) has concluded that the weight of evidence suggests that chloroform does not have direct genotoxic potential (WHO 2004), but the European Agency for Safety and Health at Work has established indicative occupational skin exposure limit values for chloroform at 10mg $(m^3)^{-1}$ or 2 mgl^{-1} per 8 working hours (EASHW 2006).

Chloroform is still used in endodontics as a solvent of root canal sealers and gutta-percha in cases of root canal retreatment (Martos *et al.* 2011) as well as for the customisation of master gutta-percha cone during root canal obturation (Van Zyl *et al.* 2005). Despite the potentially serious concerns regarding its safety due to dental personnel inhalation exposure risks (Allard & Andersson 1992), risk assessment studies report that chloroform has no negative health effects under careful and controlled use (Margelos *et al.* 1996, Chutish *et al.* 1998). The results of this study showed that chloroform formation is evident with all combined components in our experimental group samples and further risk assessments are essential in dentistry.

The results of this study offer an overview of the chemical interactions within chlorinated aliquots of endodontic origin. The detection of VOCs and DBPs and their relevant concentrations

should be interpreted with caution as they do not entirely represent conditions of endodontic practice. However, they do indicate that additional chemical compounds that are encountered when NaOCl is used as main root canal irrigant. So, the outcomes of this study cannot be directly extrapolated in clinical conditions, but the formation of DBPs and VOCs implies that the risks and drawbacks from the use of NaOCl in dental clinical procedures require critical review and further appraisal with respect to human and environmental exposure.

Despite the use of safety measures such as rubber dam, face masks and visors, the patients and more so the dental staff and may accidentally inhale the volatile phase of DBPs formed during the contact of NaOCl with organic substrates in chemomechanical and irrigation procedures. Their exposure to chlorinated disinfection by products represents a potential threat to human health, as they tend to accumulate in human adipose tissue (ATSDR 1997). There is also a risk of inadvertent extrusion of toxic chlorinated compounds into the periapical space and blood circulation during root canal preparation (Tanalp & Güngör 2014). In addition, the aspiration of chlorinated aliquots with dental unit surgical suction during root canal irrigation may contribute to the accumulation of DBPs as liquid suspensions in sewers and waste-water distribution systems in dental surgeries or hospital premises. Further studies are required for the examination of the cumulative effects on dental staff and the degree of patient exposure through accidental inhalation of the volatile phase of DBPs during endodontic treatment procedures, under conditions of good practice.

3.5 Conclusion

SIFT-MS is shown to be an effective technique for the real-time analysis of volatile compounds released by the reaction of NaOCl and representative components of an infected root canal system. Within the limitations of this *ex vivo* study and in the rejection of the null hypothesis, the chemical interaction of NaOCl 2.5% with dentine powder, bacteria, bovine serum albumin and their combination resulted in the formation of toxic DBPs and VOCs under both aerobic and anaerobic conditions.

Chapter 4

Inadvertent apical extrusion of volatile compounds and disinfection by-products, during chemo-mechanical preparation of infected root canals: A SIFT-MS analysis

4.1 Introduction

Chlorination of water for disinfection purposes may result in the formation of potentially harmful DBPs, due to the reaction of chlorine or hypochlorite with NOM (Nikolaou *et al.* 1999, Krasner 2009, Chen & Westerhoff 2010). In the presence of DBPs, daily exposure to chlorinated water via inhalation, drinking, dermal absorption through bathing, showering and swimming (Lin & Hoang 1999, Backer *et al.* 2000, Ashley *et al.* 2005, Chowdhury *et al.* 2014), may be harmful to human health because of the carcinogenic and mutagenic properties of these DBP compounds (Villanueva *et al.* 2006, Richardson *et al.* 2007).

The use of NaOCl has been universally adopted in endodontics as the main irrigant for the disinfection of infected root canals (Sedgley 2004). A consensus exists that a concentration of 2.5% NaOCl is clinically acceptable both in terms of antimicrobial and dissolution capacity in endodontic treatments (Basrani & Haapasalo 2012). The use of a chelating agent, such as 17% EDTA, aids in the removal of the infected inorganic components of the forming smear layer, during root canal instrumentation (Haapasalo *et al.* 2012).

As mentioned previously in Chapter 3, the formation of harmful VOCs and chlorinated DBPs occurs during treatment of dentine with hypochlorites. Varise *et al.* (2014) reported the detection of organochlorine compounds including chloroform, hexachloroethane, dichloromethylbenzene and benzaldehyde, after 15min interaction of 0.5%, 2.5%, 5.25% NaOCl with bovine dentine powder and pulp tissue fragments, using GC-MS. In Chapter 3, the results showed that the 30min chemical interaction of 2.5% NaOCl with combined sources of infected root canal content including dentine powder, planktonic multi-microbial suspensions and bovine serum albumin resulted in the formation of increased levels of toxic VOCs and DBPs, such as ammonia, acetaldehyde, ethanol, acetonitrile and chloroform, with the aid of SIFT-MS. The emergence of

toxic VOCs and chlorinated DBPs from the interaction of NaOCl with infected root canal content requires further examination due to the potential hazards during root canal preparation and irrigation.

The risk of debris extrusion into the periradicular tissues through the apical foramen, during chemomechanical preparation can occur even under conditions of strict control of the root canal length and good clinical practice (Tanalp & Güngör 2013). Hülsmann *et al.* (2009) stated that in any endodontic treatment, an instrument used in an apical direction or an instrument acting as a plunger may result in periapical extrusion of root canal biomass. NaOCl is a relatively toxic chemical that cause significant damage to periapical tissues even with low-scale exposure in terms of time, quantity and concentration (Pashley *et al.* 1985, Kerbl *et al.* 2012). Kerbl *et al.* (2012) reported degradation of the organic matrix in dog femur bone when in contact with 5.25% NaOCl, that led to significant changes in the cancellous structure, leaving large craters of apparent demineralisation and paucity of cancellous bone trabeculation and bone marrow. Furthermore, even dilute solutions of NaOCl can cause hemolysis, skin ulceration and eye irritation (Pashley *et al.* 1985). However, apart from NaOCl, any source of combined chemical, physical, mechanical, microbial irritation has a potential to disrupt the integrity and balance of periradicular tissues, leading to the induction of periapical inflammatory response (Siqueira 2003). From a clinical point of view, the occurrence of apical extrusion in root canal procedures may predispose for the occurrence of inter-appointment flare ups, post-operative pain, extra-radicular infection and delayed healing response (Siqueira 2003, Ng *et al.* 2008).

NaOCl is a strong oxidising agent and interacts with the infected content of the root canal system, whilst instrumentation progresses and irrigation depth increases. Disrupted microbial biofilms, vital or necrotic cells, blood and plasma exudates, vital or necrotic pulp remnants comprise this multivariable content (Torabinejad *et al.* 2002) and provide a constant reservoir of natural organic matter (NOM), exposed to replenishing volumes of NaOCl. The forming post-chlorinated biomass may potentially extrude through the root apex during root canal chemo-mechanical preparation. Hence, any forming VOCs and chlorinated DBPs may penetrate and leak through the apical foramen, biologically interact with periapical tissues and flow through blood circulation. However, the identification and quantification of VOCs and chlorinated DBPs as well as the risk of extrusion in the periradicular space have not been elucidated yet.

The aim of this study was to screen and quantify *ex vivo*, the formation and extrusion of VOCs and chlorinated DBPs in a water-closed periradicular space, following the clinical simulation of instrumentation and irrigation of infected root canal specimens, with rotary NiTi instruments, 2.5% NaOCl and 17% EDTA. The null hypothesis was that the chemo-mechanical preparation of *ex vivo* infected root canals did not result in the formation and extrusion of VOCs and chlorinated DBPs.

4.2 Materials and Methods

4.2.1 Sample size calculation

A two-way repeated measures experimental design was employed. Sample size estimation was conducted a priori with G*Power 3.1.9.2 software (Franz Faul, Universitaet Kiel, Germany). To ensure that a standardized effect of size 0.23 would be detected by two-way ANOVA at 80% power and with a probability of alpha-type error of 0.05, a sample size of 42 specimens was required for three experimental groups (n=3x14). To compensate for uncertainty in these assumptions, 4 additional specimens were added in each group, resulting in a total of 54 human single-rooted teeth for use in the study.

4.2.2 Specimen selection and preparation

Fifty-four (N=54) freshy extracted single rooted teeth with a single round canal, that were free of cracks, fractures, caries, external cervical root resorption, abrasions and discoloration were collected. Informed and written consent was obtained by medically-fit patients, who were referred by their dentists to have their teeth extracted in dental surgery premises. The collection and specimen storage procedures were approved and conducted in accordance with the protocol outlined by the Research Ethical Committee (Wales REC 4, 14/WA/1004, UK).

All specimens were soaked into 0.5% NaOCl for 60min to remove soft tissue and root surfaces were polished with elastic pumices. The crowns of the teeth were removed with a wafering blade and the length of each root specimen was standardised to 15mm. Digital

radiographs (Digora Optime, Acteon, UK) were taken in buccal and proximal directions. Radiographic criteria for tooth selection were the presence of a single canal, root curvature <10^{o}, no signs of internal and external apical root resorption or calcification and a fully developed apex.

To ensure apical patency, a sterile size 8 K-file (Dentsply Sirona, Switzerland) was initially placed 1mm beyond the foramen. The working length of each specimen was determined by apical insertion of a size 10 K-file (Dentsply Sirona, Switzerland) in the canal until its tip was detected through apical foramen, followed by 1mm subtraction. All procedures were carried out under magnification with a dental operating microscope (Global Surgical Corporation, USA). The root canals were initially prepared up to working length, with ProTaper Universal (Dentsply Sirona, Switzerland) rotary files S1, S2 and F1. Irrigation was performed with 5.25% NaOCl (Chloraxid, Cerkamed, PL) using a total volume of 6ml (2ml per file sequence). The prepared root canals were flushed with 2ml distilled water and 2ml 17% EDTA (Schottlander & Davis, UK) were used to remove the inorganic phase of the smear layer. A final flush with 2ml distilled water was performed to remove any irrigant residues and the canals were dried with sterile paper points (Size F1, Dentsply Sirona, Switzerland).

4.2.3 Fabrication of testing apparatus

A novel testing apparatus was fabricated to simulate the conditions of mechanical preparation and intracanal irrigation in a 'water-closed' apical system, to reflect periapical tissue resistance to irrigant extrusion and simulate high- compliance periapical lesions (Psimma *et al.* 2013). A mini zinc plated fuel hose line clamp (diameter:11-13 mm, height:10 mm) (Wilson Lendrum & Weir, Northern Ireland, UK) was initially adjusted and fitted 2mm in depth (1/5 of actual height) to the external diameter (11.5-12mm) of the open end of a 4ml clear glass bottle (Ampulla, Cheshire, UK) (Figure 4-1).

Each root specimen was vertically stabilised on its coronal surface with thermo-plasticised silicone glue with the aid of a glue gun (Bosch PKP 18 E, Robert Bosch Holdings, UK). Every clamp was positioned in the periphery of each root to achieve a centering position of specimen within the diameter of the clamp margins. The clamp was then filled with same type injectable thermo-plasticised silicone glue (Bosch PKP 18 E, Robert Bosch Holdings, UK). The root

specimen was covered up to apical third by silicone glue, which was cylindrically shaped after application of vertical pressure with the aid of each 4ml clear glass bottle (Figure 4-1). The apical 3mm remained exposed within the boundaries of the glass vial. The testing apparatus consisted of two elements: the silicone-infused clamp, as a representative mold of the external surface of each root specimen, and the selected 4ml glass vial. The clamp was tightened with the aid of an electric screwdriver (Bosch IXO, Robert Bosch Holdings, UK), to ensure the performance of the apparatus as a single unit. Once the materials set, the root specimen was removed and the apparatus was inspected to ensure that no structural deficiencies were evident. Prior to use, every surface of the testing apparatus elements was sterilised under UV irradiation for 2h.

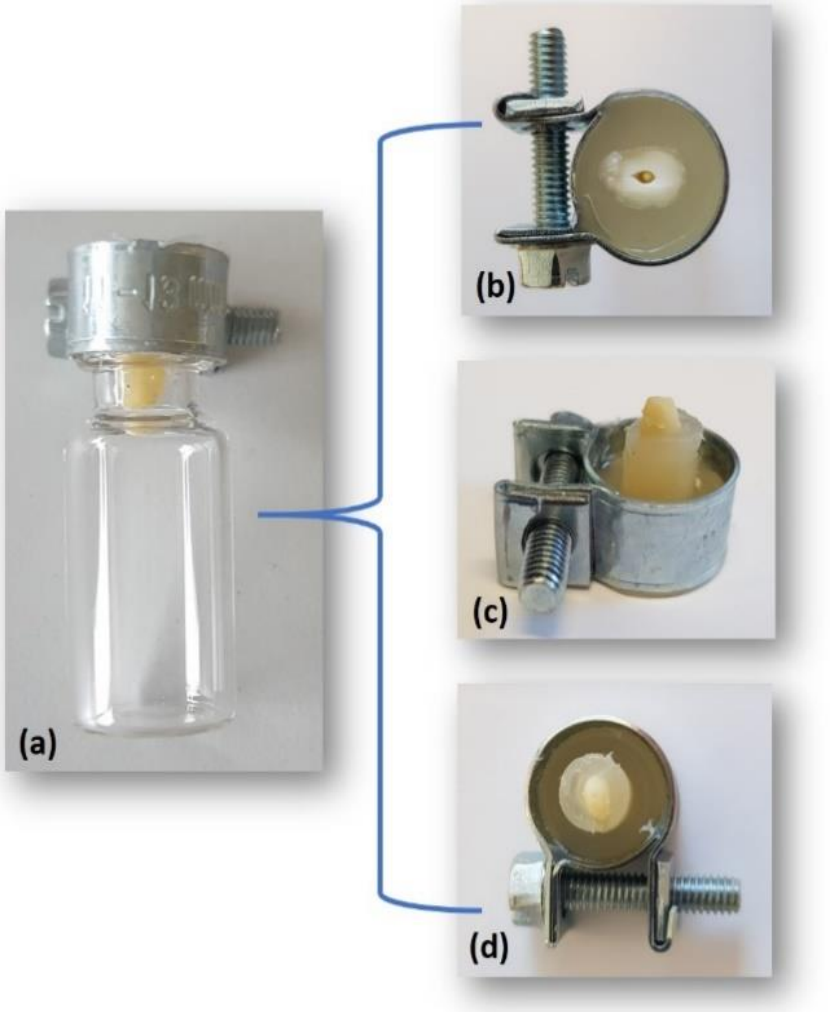


Figure 4-1 Infected tooth-model testing apparatus. (a) Adjustment of clamp diameter to glass bottle open end. (b) Coronal view of clamp-silicone index. (c) Lateral view of clamp silicone index. (d) Apical view of clamp-silicone index.

4.2.4 Development of nutrient-stressed multispecies biofilm within root specimens

A stressed multispecies biofilm comprising of five selected bacteria was developed on the root canal of each selected hemi section using the protocol developed by Niazi *et al.* (2014). The selected endodontic bacteria in this biofilm included *Propionibacterium acnes, Actinomyces radicidentis, Staphylococcus epidermidis, Streptococcus mitis* -recovered from root canals of teeth with refractory endodontic infections- and *Enterococcus faecalis* strain OMGS 3202 (Dahlén *et al.* 2010, Niazi *et al.* 2010).

To establish the biofilms, the strains were cultured anaerobically at 37°C for seven days on Fastidious Anaerobe Agar supplemented with 5% defibrinated horse blood (FAA, Thermo Scientific™, UK). Individual starter cultures of each species were collected with inoculation loops (Cole-Palmer, UK), added in filter-sterilized modified fluid universal medium (mFUM) (Gmür *&* Guggenheim 1983) and incubated at 37°C for 3h in anaerobic workstation (MACS-MG-1000, Don Whitley Scientific Ltd, UK). The absorbance was adjusted with fresh mFUM to 0.5 at 540nm to obtain 10^7 cells/ml per specie (Labsystems iEMS Reader MF, Basingstoke, UK).

The prepared root specimens were autoclaved at 121°C for 15min and placed in sterile 2ml containing Eppendorf safe-lock tubes (Eppendorf UK, Stevenage, UK). The root canals and the tubes were filled with mFUM and pre-reduced in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% carbon dioxide) for 2h and then mFUM was aspirated. Six, randomly selected, sterile root specimens remained in mFUM, without microbial inoculation to confirm sterility and no biofilm growth.

The root canals of the remaining 48 specimens were filled to the orifice level with a suspension, containing a mixture of the five microbial species of equal volume, with the aid of sterile 1-ml insulin syringes with a 30-G needle (Medisave, UK). Prior to incubation, the canals were gently hand instrumented with a sterile 15 K-file (Dentsply Sirona, Switzerland) up to working length to carry the bacteria down to the length of the canals. The Eppendorf safe-lock tubes were then re-filled with mFUM. The biofilms were grown anaerobically with regular medium change after every 24h, for the first 7 days. In the next 7 days, the biofilms were left to grow in the same unchanged medium in order to stress microorganisms nutritionally (Niazi *et al.* 2014). After a total

period of 14 days, the 48 biofilm-containing root specimens were prepared for assignment to one control and two experimental groups (n=16 specimens/group).

4.2.5 Examination of biofilm development

After 14 days, the six sterile root specimens (no biofilm growth) and six randomly selected inoculated root specimens (two per group; biofilm growth) were prepared for scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) examination. A low speed abrasive diamond disc was used to cut a groove along the long axis of each specimen without reaching the root canal system (TOC, Bristol, UK) (Lin *et al.* 2013). A chisel (Draper Tools, Hampshipe, UK) was then used to split the tooth open into two pieces (Al Shahrani *et al.* 2014). These procedures were performed in a Class II laminar flow biological safety cabinet (Nuaire, USA), to avoid cross-contamination of the specimens.

For SEM imaging, the dissected specimens were immediately fixed in phosphate-buffered formalin 10%, dehydrated in ethanol and dried in hexamethyl disilazane (HMDS) (Agar Scientific, Stansted, UK) (Bhuva *et al.* 2010). The specimens were gold sputter coated (Polaron E5100; Quorum Technologies, Ringmer, UK) and used for SEM examination (JCM-6000PLUS NeoScope Benchtop SEM, Jeol, USA) to confirm the absence/establishment of the biofilm, at a magnification of x500-x2000, operating at 10kV.

For CLSM imaging, the biofilms were stained with a Live/Dead Baclight bacterial viability kit (Thermofisher Scientific, UK) and visualized under a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Milton Keynes, UK). A x63 magnification oil immersion objective with a numerical aperture of 1.40, and a confocal pinhole to Airy 1 unit was used to observe the fluorescence emission of SYTO® 9 and Propidium Iodide using 488nm and 569nm (Ar-Kr laser) as the excitation source, respectively. Image acquisition was performed with a zoom factor of 4.0, a pixel resolution of 0.11μm/pixel and field resolution of 512x512pixels.

4.2.6 Protocols of chemo-mechanical preparation procedures

After 14 days, the root specimens were removed from the inoculation tubes and the external root surfaces were dried with sterile gauzes. They were then transferred into their assigned silicon indices. The glass vials were filled with 4ml sterile ultrapure water (Simplicity UV Milipore SAS, France), merged with the clamps and tightened with the aid of an electric screwdriver (Bosch IXO, Robert Bosch Holdings, UK), to ensure the performance of the apparatus as a single unit. A PTFE white thread tape (0.075mm thickness) (RS Pro, UK) was further adjusted around their external interface to ensure air-tight marginal adaptation, thus further sustainability of a 'water-closed' periradicular system and maintenance of apical pressure (no pressure equalisation). The glass vials were also coated with a brown packing tape (Packatape, UK) to ensure no visualization of the root apex and the periradicular space by the operator.

The remaining 42 infected root specimens were randomly divided into 3 Groups (n=3x14), using a software (List Randomiser) (https://www.random.org/lists/). In Group 1, no endodontic intervention was performed initially. Groups 2 and 3 were the experimental groups, in which endodontic intervention was performed in a Class II laminar flow biological safety cabinet (Nuaire, USA), to prevent cross-contamination of the specimens.

Mechanical root preparation was conducted with the use of rotary files Protaper Gold Universal instruments F1, F2 and F3 (Dentsply Maillefer, Switzerland). The files were connected with a 6:1 contra-angle hand-piece in an endodontic-motor (X-Smart Plus Endo Motor, Dentsply Maillefer, Switzerland), set at speed=300rpm and torque=3Ncm^{-1}. Each file was used in a crown-down manner, with in-and-out brushing motion against all root canal walls, gradually progressing at full working length, after 30s of use. The irrigants used were sterile saline (NaCl 0.9%) (JFA Medical, Blackpool, UK), 2.5% NaOCl and 17% EDTA (Schottlander & Davis, Letchworth Garden City, UK).

Concentrated NaOCl solution was prepared from a stock solution NaOCl ≥10% (Sigma Aldrich, Gillingham, UK) and verified with a standard iodine/thiosulfate method (iodometric titration) (Vogel 1962). Syringe irrigation was performed using a 27-Gauge, open-ended needle and a 3ml containing syringe with a luer-lock (Monoject, Medtronic, UK). A rubber-stop was applied on the needle, 3mm short of working length. Irrigation was carried out by using digital

pressure with the forefinger only at an estimated flow rate 3ml.min^{-1}. The needle was gently moved up and down in a 2mm range, ensuring that it did not bind on axial walls.

Ultrasonic activated irrigation (UAI) was performed with application of a size 25 and zero taper ultrasonic file (Irrisafe, Acteon, UK) in the filled with irrigant root canals. The ultrasonic unit (Newtron Booster, Acteon, UK) was adjusted for endodontic use (ring colour at yellow) with a power setting at 9, according to the manufacturer's instructions. The file was inserted into the canal 1mm short of working length.

Group 1 (n=14) received no endodontic intervention whilst chemo-mechanical preparation in Groups 2 and 3 was performed by an accredited Specialist Endodontist. The sequence of instrumentation and irrigation procedures for Groups 2 and 3 is described in Figure 4-2 and briefly summarized below:

- Group 2 (n=14): Protaper F1+3ml saline, Protaper F2+3ml saline, Protaper F3+7ml saline, 1ml saline+UAI.
- Group 3 (n= 14): Protaper F1+3ml 2.5% NaOCl, Protaper F2+3ml 2.5% NaOCl, Protaper F3+3ml 2.5% NaOCl, 1ml saline / 2ml 17% EDTA / 1ml saline, 1ml 2.5% NaOCl / UAI.

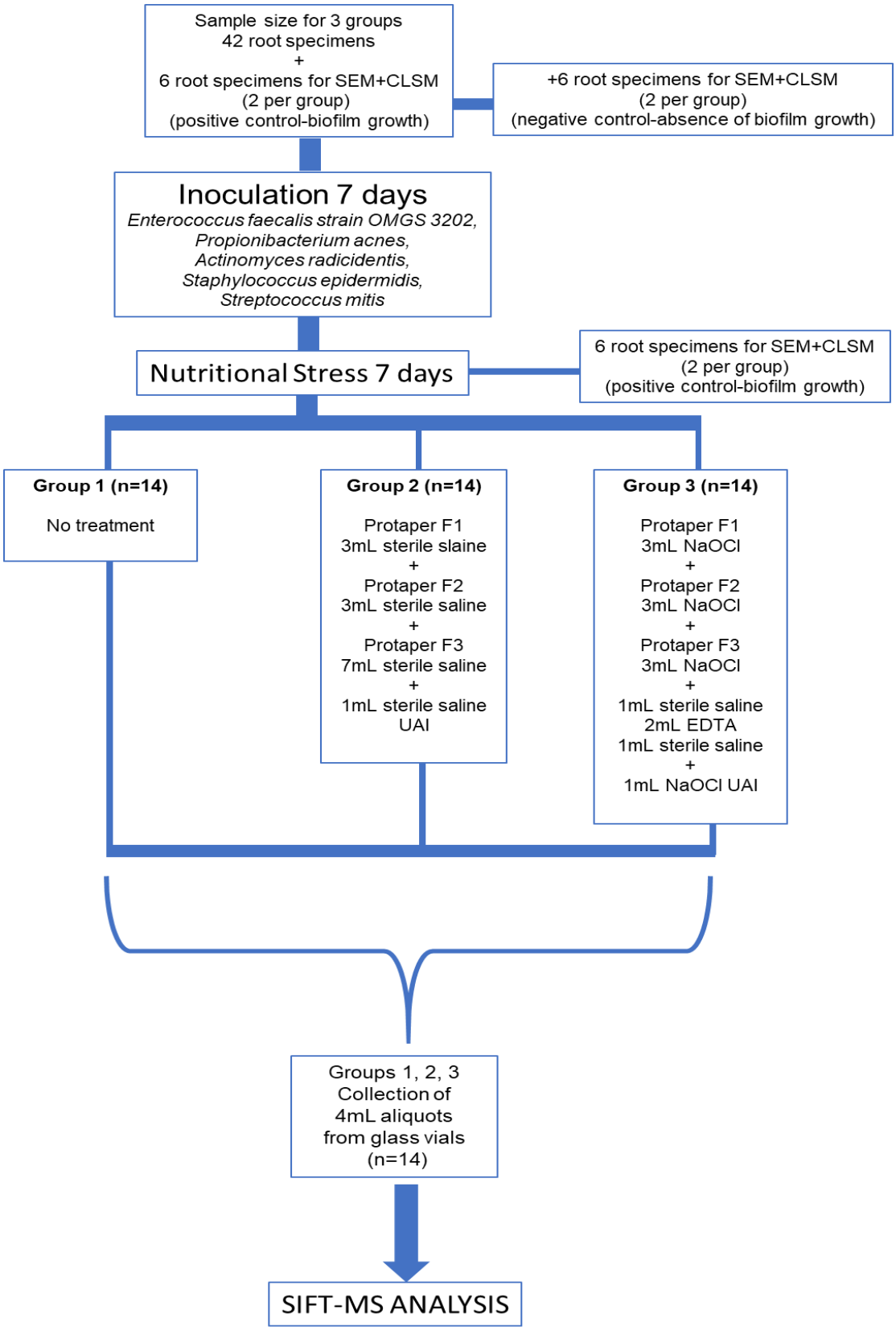


Figure 4-2 Flow-chart of designed experimental procedures, including sample size calculation, allocation of root specimens, biofilm growth, protocols of root canal chemomechanical preparation and SIFT-MS analysis.

During irrigation, a portable medical suction unit (Armoline, Medical Import London, UK) was used to aspirate the effluent from the prepared root canals, with aid of a sterile silicon tube (external diameter:10mm; wall thickness:2mm) (Ad Fontes Company, Hong Kong), connected with autoclavable polycarbonate liquid collection jars, assigned for each root specimen separately (Figure 4-3). The use of dental suction was contributory to the simulation of clinical conditions and standards of good endodontic practice.

Once chemo-mechanical procedures were complete, the clamp-silicone-specimen apparatus was untightened from the glass vial. The liquid content (4ml) of the 'water-closed' periradicular space obtained from Groups 1, 2 and 3 (Figure 4-3) was dispersed in sterile polystyrene bijou containers (Sterilin, Thermo Scientific™, UK) and kept refrigerated at -80ºC until analysis.

In order to obtain a better knowledge of all possible occurring chemical interactions, we also mixed 1ml NaOCl 2.5%with 1ml EDTA 17%, obtained from 5 different commercially available brands for dental use, in the absence of dentine and bacterial components. The aliquots were stirred for 3min and kept refrigerated at −80◦C until analysis.

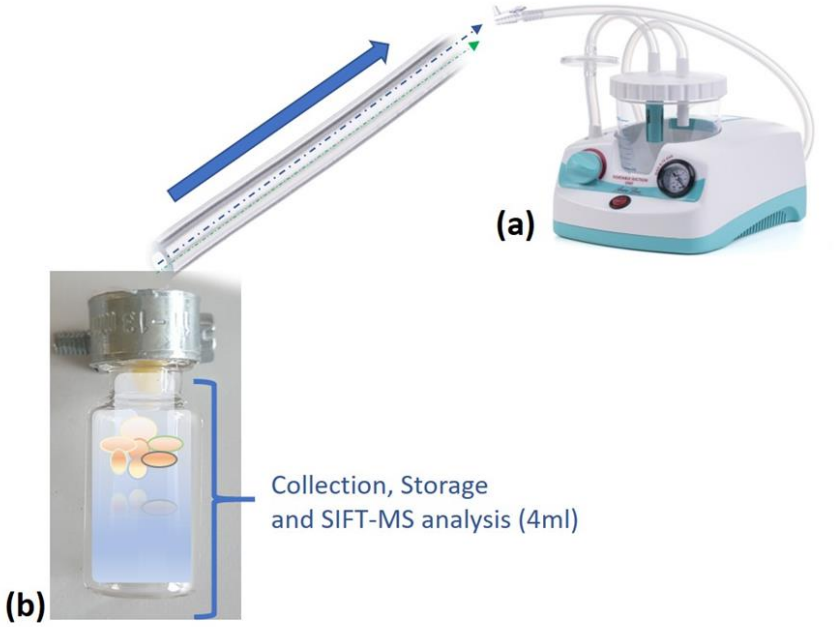


Figure 4-3 Experimental set-up of root canal irrigation and aspiration. (a) Application of portable medical suction. (b) Water-closed apical system filled with sterile ultrapure water.

4.2.7 SIFT-MS analysis

The SIFT-MS is a quantitative mass spectrometry technique used for trace gas analysis and has been described in detail elsewhere (Smith & Španěl 2005) however, a brief explanation is presented. In SIFT-MS, a mixture of reagent ions (H_3O^+, NO^+ and O_2^+) are generated in a microwave discharge. Each of these reagent ions can be selected by a quadrupole mass filter and separately injected into a fast-flowing helium carrier gas in a flow tube. The sample gas to be analysed naturally flows into the helium at a controlled rate via a calibrated capillary by virtue of the atmospheric pressure of the sample gas and the much lower pressure of the helium (typically 1mbar). The chosen reagent ion then reacts with the trace components in the sample (to the exclusion of the major air components) to generate product (analyte) ions. The reagent ions and analyte ions are mass analysed by a quadrupole mass spectrometer and counted by a detector. Thus, the characteristic analyte ions identify the neutral trace components present in the sample and their count rates provide their concentrations in real time.

The SIFT-MS instrument may be operated in two modes: the scan mode where a whole spectrum is captured over a desired m/z range, or a selected ion mode, where individual compounds are targeted and analysed individually. The former is ideal for identifying compounds of interest; the latter produces more accurate quantification. Both modes were used in this study, but quantification was carried out using the selected ion mode.

For analysis, the samples were defrosted in air. Analysis of the headspace volatile compounds was carried out in real-time by SIFT-MS. Prior to analysis, three replicate 1ml aliquots of each sample were placed into a sample bag constructed from 50cm length, 65mm diameter Nalophan NA (Kalle, UK), which was then filled with purified air and sealed prior to incubation at 37^{o}C. After equilibrium between the liquid and headspace above it (30min), the headspace was sampled directly into the SIFT-MS via a heated, calibrated capillary that defines the headspace sample flow rate, as is necessary for absolute quantification of VOCs. The analytical downstream quadrupole mass spectrometer was scanned over the range of mass-to-charge ratio, m/z, using the three reagent ions H_3O^+, NO^+ and O_2^+ independently. In addition, individual compounds of interest were selected and the product ions from these compounds were targeted and analysed. From the m/z values of the analyte ions and their count rates, and using the kinetics database

stored in the instrument library, the concentrations of the identified VOCs were immediately obtained (Smith & Španěl 2005, Španěl & Smith 2011).

4.2.8 Statistical Analysis

Two-way analysis of variance (ANOVA) with post hoc Tukey tests was used for data analysis of the forming VOCs and DBPs from the periradicular space. The overall analysis was performed with SPSS software (version 22.0, IBM SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P<0.05$.

4.3 Results

4.3.1 Biofilm growth in root specimens

The examination of the infected tooth specimens with SEM confirmed the presence of a thick biofilm layer, attached on the main canal lumen as well as extending within dentine tubules (Figure 4-4). The images obtained from confocal analysis, confirmed the presence of live microbial cells (green) as a major proportion of the grown biofilm, as well as minor proportions of dead microbial cells (red), as a sequence of biofilm metabolic process (Figure 4-5). On the contrary, the examination of the sterile tooth specimens showed the presence of open dentine tubules and confirmed the absence of microbial populations (Figures 4-4c, 4-5c).

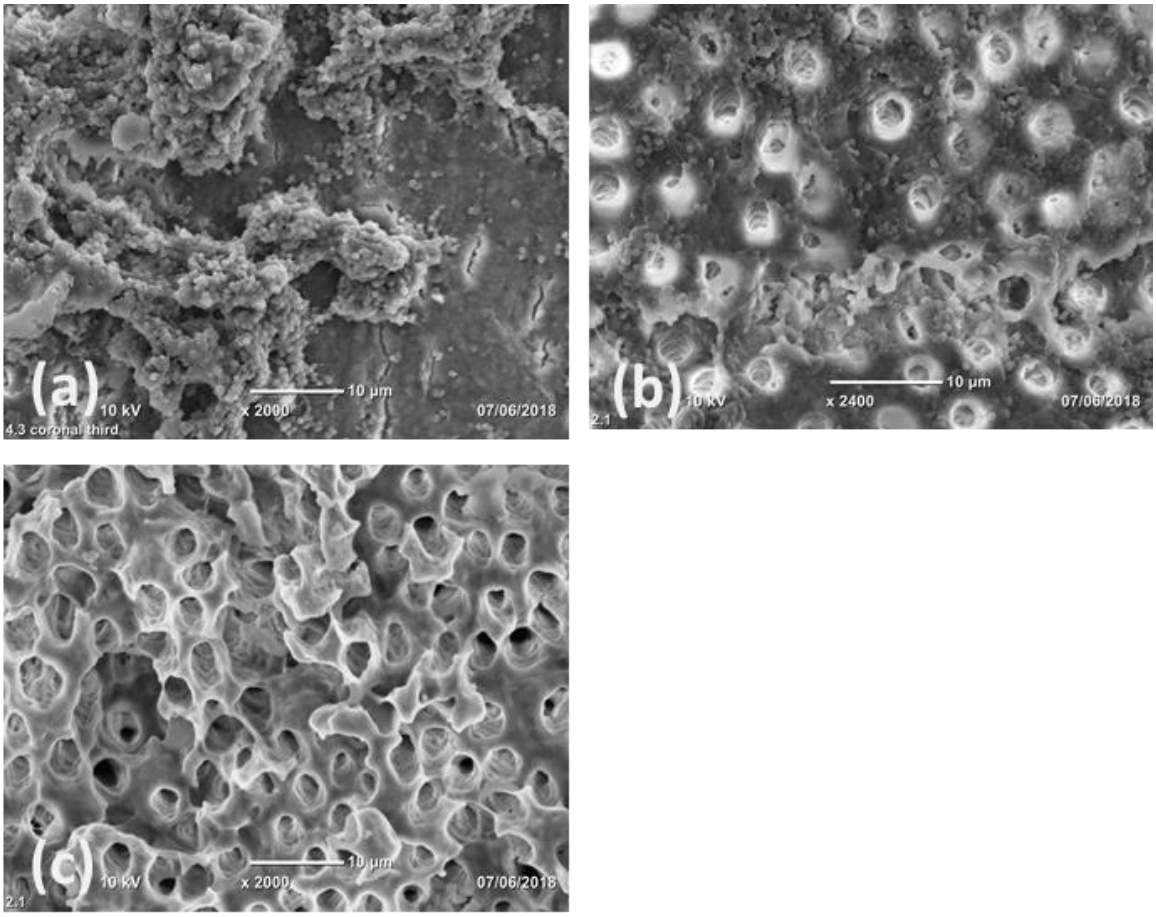


Figure 4-4 SEM examination of infected and non-infected tooth specimens. (a) Biofilm growth attached in intra-radicular dentine surface, facing main root canal lumen; (b) Biofilm growth within dentine tubules; (c) Sterile, patent and bacteria-free dentine tubules.

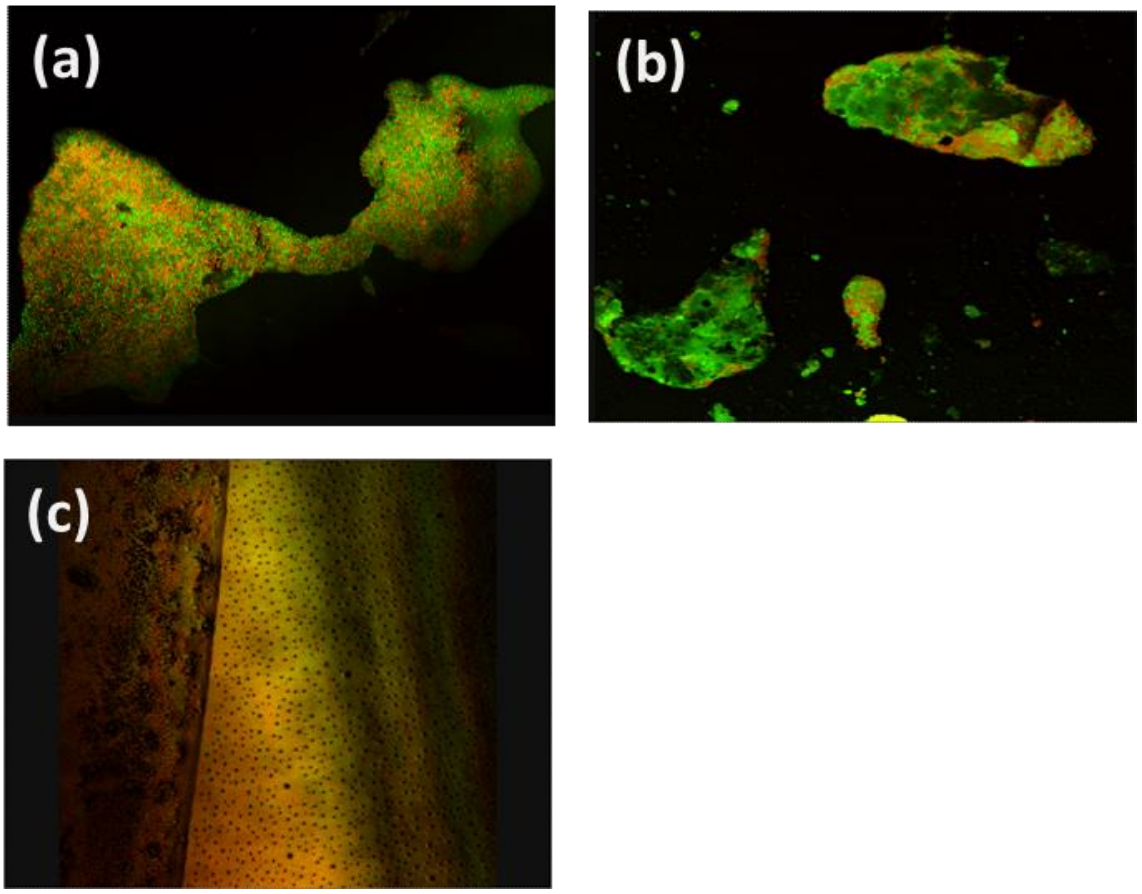


Figure 4-5 CLSM examination of infected and non-infected tooth specimens. (a) Biofilm growth attached in intra-radicular dentine surface, facing main root canal lumen; (b) Biofilm growth within dentine tubules; (c) Sterile, patent and bacteria-free dentine tubules.

4.3.2 SIFT-MS analysis of aliquots from periradicular space (apical extrusion)

The headspace of all samples, following treatment procedures in Groups 2 and 3, contained several common compounds that are often seen in biological media headspace, including acetone, acetic acid, methanol, ethanol, propanol, acetaldehyde, acetonitrile and ammonia (Table 4-1) (Turner *et al.* 2008).

The concentration of all compounds in Group 2 were detectable, but at very low concentrations in the parts-per-billion by volume (ppb.v) range. In Group 3, the concentration of all compounds, except for ethanol, were present at much higher concentrations, as can be seen by the very busy spectra shown in Figures 4-6 and 4-7. The pronounced peak at m/z 31, in Figure 4-6, is due to formaldehyde and the 3 peaks at m/z 83, 85 and 87, in Figure 4-7, are the

isotopologues of chloroform (Španěl & Smith 1999). These compounds are at statistically significant headspace concentrations compared to those in the headspace of Group 2 ($P<0.05$).

With the exception of acetonitrile, a large increase in concentrations from ppbv to parts-per-million by volume (ppm.v) scales of measurement was seen in Group 3 for methanol, propanol, ammonia, chloroform and formaldehyde. No formation of VOCs or DBPs occurred in Group 1 headspace, when chemo-mechanical preparation was not performed.

Table 4-1 Mean (SD) concentrations (ppb.V) of VOCs and DBPs after SIFT-MS analysis of aliquots obtained from periradicular space.

Aliquots in periradicular space (4 ml) **Volatile compounds**	**Group 1** **No endodontic intervention (n=14)**	**Group 2** **Chemomechanical preparation with Distilled Water (n=14)**	**Group 3** **Chemomechanical preparation with 2.5% NaOCl and final flush with 17% EDTA (n=14)**
Acetone	N/D	7.32 (7.23) [a]	27.18 (14.86) *
Acetic Acid	N/D	49.36 (23.61)	144.71 (57.27) *
Methanol	N/D	96.1 (54.15)	**4903.37 (2813.75) ***
Ethanol	N/D	139.81 (70.21)	202.44 (80.12)
Propanol	N/D	17.71 (24.35) [b]	**2462.22 (1459.26) ***
Acetaldehyde	N/D	17.64 (6.72)	**440.43 (138.27) ***
Acetonitrile	N/D	5.86 (3.35)	53.79 (29.08) *
Ammonia	N/D	416.21 (93.26)	**3620.71 (1851.73) ***
Chloroform	N/D	52.07 (9.49)	**1266.64 (340.49) ***
Formaldehyde	N/D	80.78 (51.71)	**10167.94 (3388.51) ***

N/D: non-detectable; [a]: 4 samples presented 0 values; [b]: 6 samples presented 0 values; *: statistically significant increase in concentration of each volatile compound in Group 3 compared to Group 2 ($P<0.05$).

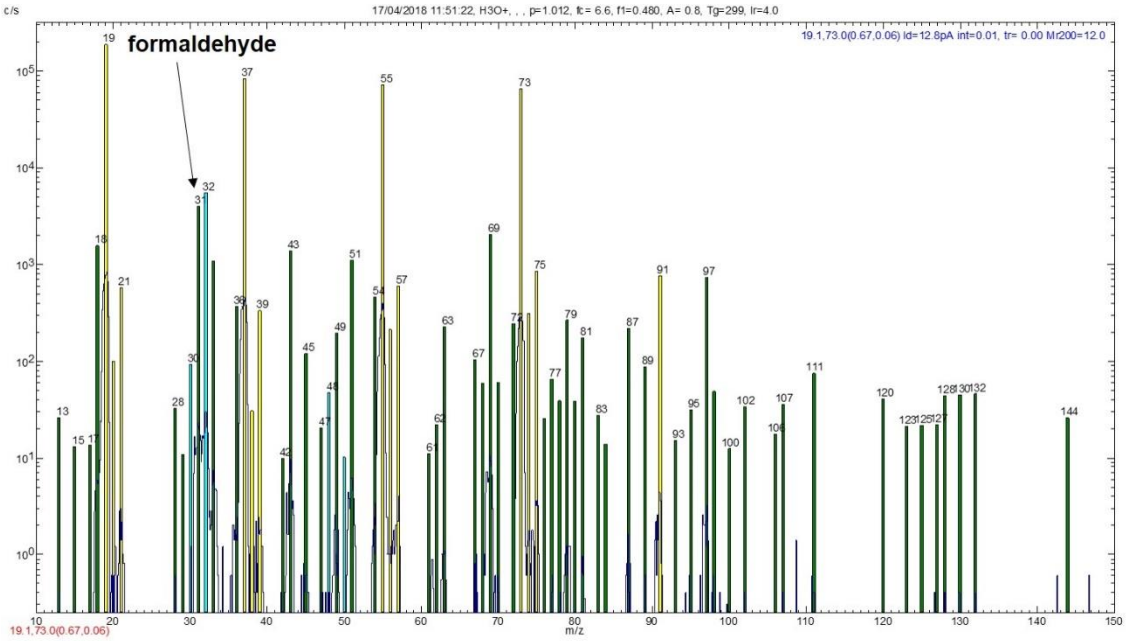


Figure 4-6 H_3O+ spectrum of Group 3 aliquot sample (irrigation with 2.5% NaOCl + 17% EDTA) obtained from periradicular space in air. Ion indicating formaldehyde at m/z 31 is shown on the spectrum.

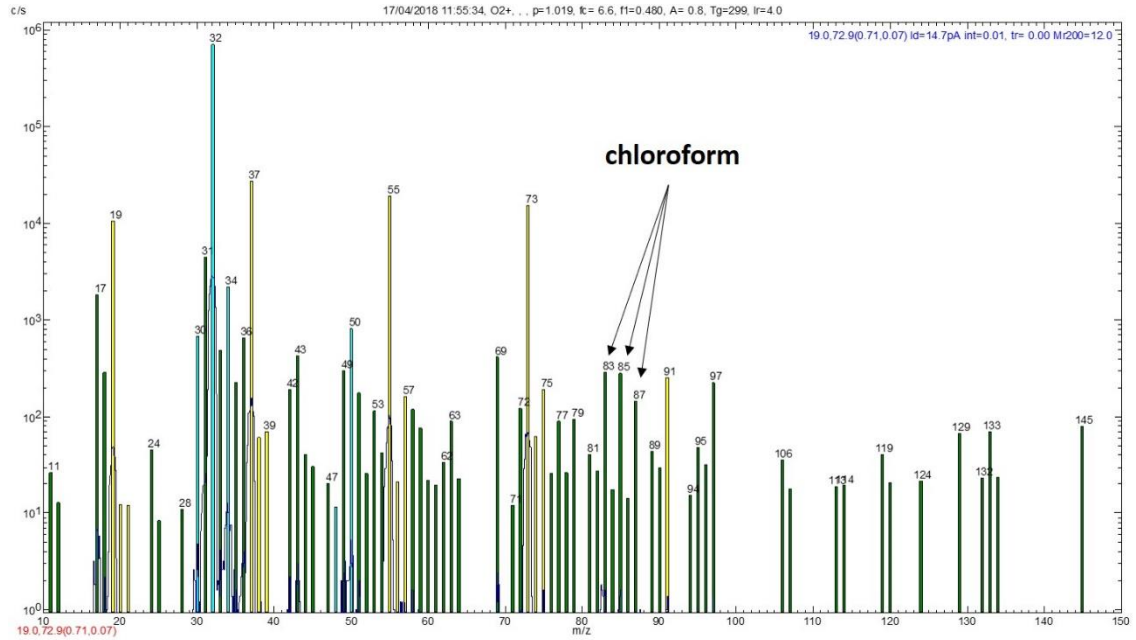


Figure 4-7 O_2+ spectrum of Group 3 aliquot sample (irrigation with 2.5% NaOCl + 17% EDTA) obtained from periradicular space in air. Ions at m/z 83, 85, 87 indicating chloroform is shown on the spectrum.

4.3.3 SIFT-MS analysis of aliquots following NaOCl-EDTA interaction

The interaction of 17% EDTA of five different brands with 2.5% NaOCl resulted in the generation of large quantities of detectable formaldehyde (Table 4-2). Furthermore, there was a strong odour of formaldehyde emanating from samples with high concentrations. The visual assessment of the mixed solutions disclosed that colour transition from transparent to brown occurred in Schottlander EDTA, with possible precipitate formation, though not visually evident (Figure 4-8a). The interaction of ENDO-solution and NaOCl resulted in complete loss of transparency and the solutions presented a greyish foggy colour (Figure 4-8b). The interaction of SmearClear and TGcleanser with NaOCl resulted in gas generation and pronounced bubble formation (Figure 4-8c, d). Finally, the interaction of Pulpdent and NaOCl did not result in any colour change (Figure 4-8e). On the contrary, all available solutions of sole 17% EDTA presented extremely low counts of formaldehyde, similar to the detection threshold of the SIFT-MS equipment (Table 4-2).

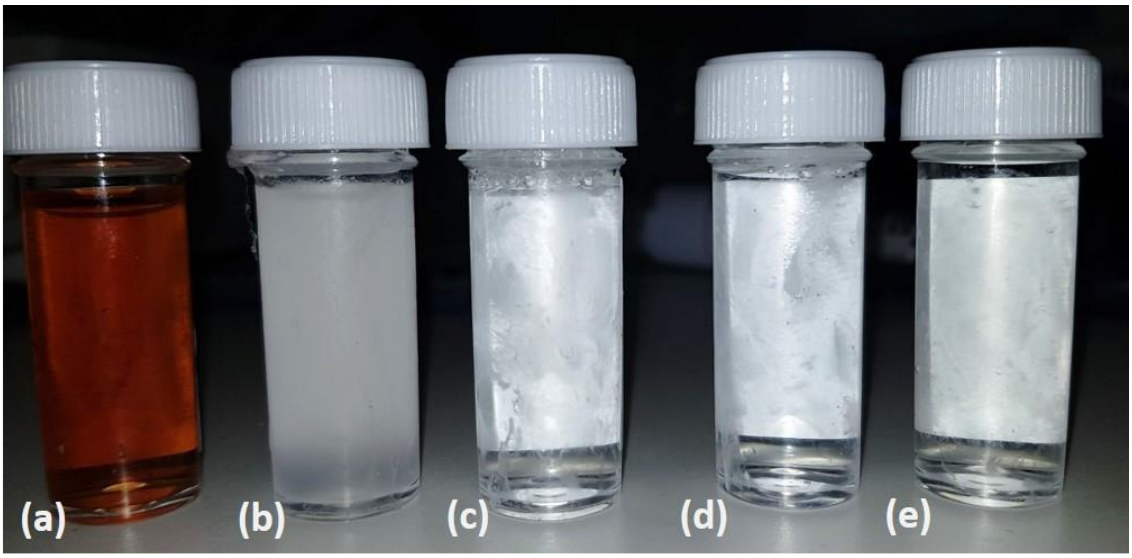


Figure 4-8 Visual assessment of the interaction of 2.5% NaOCl and different brands of commercially available 17% EDTA solutions for endodontic use. (a) Schottlander 17% EDTA + 2.5% NaOCl. (b) Endo-solution 17% EDTA + 2.5% NaOCl. (c) SmearClear 17% EDTA + 2.5% NaOCl. (d) Tgcleanser 17% EDTA + 2.5% NaOCl. (e) Pulpdent 17% EDTA + 2.5% NaOCl.

Table 4-2 Formaldehyde release following SIFT-MS analysis of 2.5% NaOCl chemical interaction with 17% EDTA of five different brands.

Product Brand / MANUFACTURER	**IRRIGANT INTERACTION (3min, 1:1 volumes)**	**FORMALDEHYDE RELEASE (ppb.V) Mean (SD)**
Schottlander EDTA (Schottlander & Davis, Letchworth Garden City, UK)	17% EDTA	155 (21)
	17% EDTA + 2.5% NAOCl	11710 (830)
Pulpdent EDTA (PULPDENT™, (Watertown, Massachusetts, USA)	17% EDTA	25 (4.9)
	17% EDTA + 2.5% NAOCl	1197 (330)
TGcleanser (TGdent, Westminster, London, UK)	17% EDTA	17.5 (5.7)
	17% EDTA + 2.5% NAOCl	333 (39)
SmearClear (Kerr™, Orange, California, USA)	17% EDTA	66 (17.6)
	17% EDTA + 2.5% NAOCl	39160 (3130)
ENDO-Solution (CERKAMED, Stalowa Wola, Poland)	17% EDTA	36 (9.0)
	17% EDTA + 2.5% NAOCl	16600 (2800)

4.4 Discussion

A novel reproducible testing apparatus was fabricated to simulate the conditions of mechanical preparation and intracanal irrigation in a 'water-closed' apical system, to reflect high-compliance periapical tissue resistance to irrigant extrusion (Psimma *et al.* 2013). The model was designed to minimize the risk of fundamental methodological limitations from the absence of periradicular tissue pressure, such as overestimation of irrigant flow or extrusion, which could potentially lead to generation of false positive quantitative data (Psimma *et al.* 2013). The potential formation of VOC's and DBP's and the incidence of periradicular extrusion using this model was conducted to simulate the *in vivo* disinfection procedure by a specialist endodontist under conditions of good clinical practice, with the adoption of a disinfection protocol that tend to minimize the risk of extrusion.

All root specimens were initially enlarged to size F1 to create a standardized apical diameter and sufficient conical taper for the inoculation of the microbial species within root canal lumen. An *in vitro* nutrient-stressed, multi-species biofilm model was successfully developed within root canals, and verified successfully with SEM and CLSM (Niazi *et al.* 2014, Niazi *et al.* 2015). The root canal system has a limited amount of oxygen and nutrients, making it a very selective environment with conditions leading to it being uninhabitable for most microbial species (Chávez de Paz 2007). The major cause of refractory endodontic infections is the persistence of bacteria in the root canal space (Moorer & Wesselink 1982). In cases of refractory endodontic infections, the survival of biofilms is due to their capacity to adjust under nutritionally stressful environmental conditions, that possibly render them resistant to the chemomechanical disinfecting procedures (Siqueira 2002). Therefore, a tooth model involving root specimens affected by a nutritionaly-stressed biofilm was utilized.

Several studies reported that the use of ProTaper rotary files at full working length caused more debris extrusion compared to other rotary systems (Bürklein & Schäfer 2012, Silva *et al.* 2016). To minimise this effect, the three finishing rotary instruments F1, F2, F3 were introduced in a coronally pre-flared root canal, then moved apically in a crown-down manner to allow for the coronal removal of infected debris, before reaching the full working length. Cervical preflaring was found to be associated with extrusion of smaller quantities of apical debris, compared to non-

cervical preflaring (Borges *et al.* 2016). A 27-G open needle was used at a safety distance of 3mm, which reduces the risk of generating apical shear stresses and extrusion (Psimma *et al.* 2013b). The application of ultrasonically activated irrigation does not pose any additional risk in terms of extrusion, in a water-closed system (Boutsioukis *et al.* 2013) and the maintenance of a low irrigant flow rate (3ml/min=0.05ml/sec) was selected to minimise the risk of irrigant extrusion (Boutsioukis *et al.* 2013). The aforementioned instrumentation and irrigation parameters were combined to ensure that that the implemented protocol of root canal chemo-mechanical preparation minimized the occurrence of extrusion resulting from the treatment protocol or the operator.

It is noteworthy to mention that during endodontic procedures the occurrence of extrusion is a combined result of all operational factors including instrumentation and irrigation. However, clinically, it is not possible to determine the frequency and amount of apical extrusion of debris or bacteria during preparation. Therefore, it may be more rational to consider the extrusion phenomenon holistically, rather than separately. Hülsmann *et al.* (2009) stated that, in any endodontic treatment, an instrument used in an apical direction or an instrument acting as a plunger may result in periapical extrusion of material, such as dentine chips, tissue remnants, microorganisms or intracanal irrigants, despite strict control of the root canal length.

The results of this study showed that the chemomechanical preparation of infected root specimens in Groups 2 and 3 resulted in the formation and extrusion of VOCs and DBPs in a water-closed periradicular space. When sterile saline was used as root canal irrigant, only low concentrations of VOCs and DBPs were detectable in the periradicular space, after chemomechanical preparation. The formation of VOCs in Group 2 is related to the metabolic activity of the growing biofilm within root canal specimens. Studies have shown that SIFT-MS technology aids in the rapid detection of small quantities of a range of VOCs, as a means of monitoring the dynamics in bacterial cultures or biofilms, in real time (Storer *et al.* 2011, Sovová *et al.* 2013, Chen *et al.* 2016). Anaerobic bacteria also form chloroform during their respiration and convert it to dichloromethane (Tank *et al.* 2013, Jugder *et al.* 2016).

The concentrations of VOCs and DBPs presented a significant increase when 2.5% NaOCl was used as the main root canal irrigant. Our findings are in agreement with the results presented

in Chapter 3. A significant increase in compounds, which are commonly present in biological media including acetone, acetic acid, methanol, propanol, acetaldehyde and ammonia was evident (Turner *et al.* 2008, Španěl *et al.* 2011, Chapter 3).

A significant increase in chloroform formation (1.2±0.3 ppm) in the periradicular space was evident in Group 3. Previous studies reported the formation of chloroform, following the interaction of NaOCl with endodontic biomaterials including dentine powder, pulp tissue fragments, planktonic multi-microbial suspensions, bovine serum albumin and their combinations (Varise *et al.* 2014, Chapter 3). In another study, the combined findings of bacterial inactivation and DBP formation confirmed that the break-down of bacterial cells provides organic precursors for DBP formation. This evidence strongly supports the pathway for DBP formation from pure bacterial biomaterials (Wang *et al.* 2013). An alternative pathway for the formation of $CHCl_3$ may also derive from the interaction of NaOCl with acetone according to the historically known haloform reaction: $3NaOCl + C_3H_6O \rightarrow CHCl_3 + 2NaOH + NaOCOCH_3$ (Reynold *et al.* 1934).

The uptake of chloroform into blood circulation via periradicular extrusion has not been examined yet, *in vivo*. The substance can be further absorbed into the body by inhalation, through the skin and by ingestion (USEPA 2001). Despite the potentially serious concerns regarding its safety due to dental personnel inhalation exposure risks (Allard & Andersson 1992), previous risk assessment studies report that chloroform has no negative health effects under careful and controlled use (Margelos *et al.* 1996, Chutich *et al.* 1998). However, considering that a new potential source of chloroform emission is present, further occupational risk assessment studies are required in dental surgeries, where large volumes of NaOCl are consumed for root canal treatments.

An unexpected finding was the formation of high concentrations of formaldehyde in Group 3, during the chemomechanical preparation with NaOCl 2.5% and EDTA 17%. This finding has not been reported in dental literature before and merits further investigation. Several studies have been performed to assess the antagonistic effects between NaOCl and EDTA, when used as root canal irrigants. In most of them, the residual free available content of NaOCl solutions was measured with iodometric titrations, when mixed with EDTA at different ratios and time intervals. The results showed that, due to an unspecified chemical reaction, the free available chlorine

content is significantly reduced; hence, the direct mixture, or the intermittent use is not recommended due to an upcoming reduction in antimicrobial capacity of NaOCl (Clarkson *et al.* 2011, Krishnan *et al.* 2017). Chlorine gas formation was also detectable and the risks of occupational exposure have been highlighted (Baumgartner & Ibay 1987, Irala *et al.* 2010, Prado *et al.* 2013). The use of nuclear magnetic resonance analysis also confirmed that a chemical reaction between NaOCl and EDTA occurs, leading to NaOCl deactivation, slow oxidation of EDTA and progressive formation of unknown by-products (Grande *et al.* 2006).

In the experimental set-up, we adopted a globally acceptable clinical protocol of root canal irrigation (Basrani & Haapasalo 2012). This involves the constant use of NaOCl during mechanical preparation with hand and rotary files and a final irrigation with EDTA to remove the inorganic phase of the produced smear layer (Basrani & Haapasalo 2012). A final flush with NaOCl under activation with ultrasonics is also considered as gold standard, in order to enhance disinfection outcomes and irrigant penetration in the open dentinal tubules (Zehnder *et al.* 2005, van der Sluis *et al.* 2007). The sole use of NaOCl, in the absence of chelation, in an infected root canal space may allow for the accumulation of infected inorganic debris (Hülsmann *et al.* 2003). On the contrary, the sole use of EDTA is not recommended due to the lack of antimicrobial properties and the risk of dentine demineralisation (Hülsmann *et al.* 2003, Haapasalo *et al.* 2014).

In addition, we took all precaution in measurements to reduce the risk of irrigant interaction. Before and after the use of EDTA the root canal was flushed with sterile saline and dried with paper points. The intermittent use of NaOCl and EDTA was not advocated due to the known consequences against dentine structural integrity and the de-activation of NaOCl (Niu *et al.* 2002, Grande *et al.* 2006). In addition, given that EDTA was used at the end of the chemomechanical preparation, we can conclude that this chemical reaction occurred in the periradicular space, after the inadvertent extrusion of chlorinated biomaterials. To ensure that formaldehyde release was not associated with any experimental errors, the same NaOCl 2.5% solution was mixed with five different brands of EDTA 17% available for dental use, at 1:1 volume ration, for 30min and SIFT-MS analysis confirmed the formation of extremely high concentrations of formaldehyde varying from 1.2ppm to 39ppm. Visual assessment of the aliquots confirmed previous findings of gas and bubble formation, colour change to brown as well as cloudy appearance.

The *ex vivo* chemomechanical preparation of a single infected root canal with rotary files, 2.5% NaOCl and 17% EDTA resulted in apical extrusion of high concentrations of formaldehyde. SIFT-MS analysis disclosed the formation of average 10ppm formaldehyde in periradicular space. Formaldehyde is a colorless, flammable gas at room temperature, which has a pungent, distinct odor and may cause a burning sensation to the eyes, nose and lungs at high concentrations (USEPA 1989). It is mainly used as a biocide in disinfection of equipment and fixation of tissue (USEPA 1989). A study conducted in Canada found that formaldehyde levels as low as 0.046 ppm were positively correlated with eye and nasal irritation in houses with urea-formaldehyde foam insulation (Broder *et al.* 1991). In 1987, the U.S. Environmental Protection Agency (EPA) classified formaldehyde as a probable human carcinogen, under conditions of unusually high or prolonged exposure (USEPA 1989). Since then, human clinical studies concluded that prolonged exposure to formaldehyde was associated with cancer of the nasopharynx and leukaemia (Hauptmann *et al.* 2003, Hauptmann *et al.* 2009, Bean Freeman *et al.* 2009). The International Agency for Research on Cancer (IARC), the Department of Health and Human Services (DHHS) and the European Chemicals Agency recently classified formaldehyde as a human carcinogen (IARC 2004, National Toxicology Programme 2011, Commission Regulation (EU) No 605/2014).

The Agency for Toxic Substances and Disease Registry (ATSDR) has established a chronic inhalation minimal risk level of 0.003ppm formaldehyde (0.004mg/m^3) based on respiratory effects in humans (ATSDR 2008). The Occupational Safety and Health Administration (OSHA) set a legal permissible exposure limit in the workplace of 0.75ppm formaldehyde in air averaged over an 8h timeweighted average work day (OSHA 2011). A second permissible exposure limit in the form of a short-term exposure limit of 2ppm, which is the maximum exposure allowed during a 15min period (OSHA 2011). The European Commission recently proposed new exposure limits for formaldehyde. An 8h time weighted average exposure limit of 0.37mg/m^3 and a 15min short-term exposure limit of 0.738mg/m^3 is now under effect (European Chemicals Agency 2018). Therefore, further occupational risk assessments are required in dental settings to justify the risk of exposure in formaldehyde and various VOCs or DBPs.

Despite the fact that the measurable concentrations exceeded the safety thresholds, in reference to occupational and patient healthcare and safety, the results should be interpreted with

caution. In this experimental set-up, one missing factor was the lack of circulation of the periradicular fluid, as a simulation of blood flow and circulation in human mandible or maxilla within cancellous bone vacuoles. Since the extruded chlorinated biomaterials remained static, the inadvertent extrusion of EDTA may have resulted in a chemical reaction of *in situ* formaldehyde generation. In human body, where blood circulation is constant, it is less likely that such high amounts may be formed. In addition, it should not be underestimated that formaldehyde can be metabolized as it is a normal component of blood. According to some studies, human body exposure to 2.4mg/m^3 formaldehyde did not increase the blood level and exposure to 0.5mg/m^3 did not result in an increase in urinary formate excretion due to rapid local metabolism (Kimbell *et al.* 2001a, Kimbell *et al.* 2001b). However, these early pre-clinical findings disclosed high levels of this toxic compound and further clinical studies are required to investigate the formation of VOCs and especially formaldehyde in the apical tissues and the systemic blood circulation in patients undergoing root canal treatment.

From the results shown here, clinicians should also consider the interaction of NaOCl and EDTA, which leads to formation of formaldehyde in gaseous form. Therefore, the intermittent use of NaOCl and EDTA during irrigation should not be recommended and this practice needs to be aborted. Our findings further raise occupational hazard concerns, as the risk of inhalation of the forming aerosol in a dental surgery is present. The patient may be potentially exposed for one or two dental sessions however the dentist and dental staff may inhale the volatile phase throughout their working days.

Taking into consideration this new implication from the combined use of NaOCl and EDTA, it is essential to review the use of EDTA and its relevant concentration for the removal of the inorganic phase of the smear layer. Alternative chelating agents, such as citric acid, must be studied further, but subject to the induction of a potential strong acid-base reaction when mixed with NaOCl. The current concept of continuous irrigation and chelation with the use of a single solution containing NaOCl and etidronic acid has showed promising results (Lottandi *et al.* 2009, Arias-Moliz *et al.* 2014). However, further investigation is required to justify the possibility of potential formation of other VOCs and DBPs.

4.5 Conclusions

Within the limitations of this *ex vivo* study, the mechanical preparation and irrigation of artificially infected root canals with rotary NiTi files, 2.5% NaOCl and 17% EDTA resulted in the formation of VOCs and DBPs in a water-closed periradicular space. The chemical interaction of NaOCl and EDTA resulted in high concentrations of formaldehyde release.

Chapter 5

VOCs and DPBs in post-chlorinated effluent aliquots following chemomechanical preparation of artificially infected root canals *ex vivo and* the role of activated carbon in their removal

5.1 Introduction

Dental professionals are under increasing pressure to understand and adhere to clinical waste regulations. Disposal and proper management of clinical waste is vital and there are strict legislations on hazardous waste for which dental practice policies are in place to prevent harm against the environment and human health (Department of Health, Environment and Sustainability 2013). Many of the dental materials that are currently in use, including heavy metals and biomedical waste present potential challenges to the environment. Of much concern in recent years has been the impact of heavy metal contamination of water systems by dentists, particularly through the production of dental amalgam waste. Dental amalgam contains mercury, silver and other metals that can generate solid and particulate wastes and contaminate the environment, if they are not properly managed (Shraim *et al.* 2011).

The aspiration of root canal irrigants with the aid of dental suction is essential for safe intracanal delivery and prevention of spillage, oral contact and ingestion of the effluent irrigant. A comprehensive literature search by the authors did not result in any studies associated with the composition and the fate of the post-chlorinated aliquots from dental unit disposal waterlines. Moreover, these aliquots have not been classified in any categories of biomedical or general office waste. During root canal irrigation and aspiration, the forming effluents definitely flow through pipelines and water distribution systems ending into drainage and sewage disposal networks, without any former point-of-use (POU) treatment or purification. The potential environmental hazards have not been determined yet. Considering the daily application of high volumes of NaOCl and EDTA in dental practices and hospitals, this appears to be potentially contributory to formation and bioaccumulation of VOCs and DBPs in liquid suspensions.

The application of enhanced coagulation, ozonation and treatment with activated carbon (AC) are among the proposed scientific methods for the elimination of VOCs, DBPs and the reduction of NOM precursors, soluble microbial precursors and humic substances (Lou *et al.* 2009, Liu & Li 2015, Zhang *et al.* 2017). The use of AC has been advocated for the removal of organic constituents, impurities and residual disinfectants in water supplies, due to its adsorption capacity (Zhao *et al.* 2018). Despite its chemical versatility, AC does not effectively dissociate bacteria and viruses, which may adhere on the surface and mesopores, using the latter as carbon source for their growth and metabolism (Kim & Park 2008). For the prevention of pathogens' growth on the AC surface and to exert antimicrobial activity, silver-impregnated activated carbon (Ag-AC) has been proposed for the POU treatment of water systems (Shimabuku *et al.* 2017). A comprehensive literature research showed no potential application of AC in dentistry or dental units for the POU pre-treatment of effluent post-chlorinated solutions that emerge from the irrigation of infected root canals.

The aim of this study was to screen and quantify *ex vivo*, the formation of VOCs and chlorinated DBPs in effluent solutions, following the clinical simulation of instrumentation and irrigation of infected root canal specimens, with rotary NiTi instruments, 2.5% NaOCl and 17% EDTA. In addition, the application of Ag-AC was investigated for the potential treatment of chlorinated effluents. The null hypothesis was that the chemo-mechanical preparation of *ex vivo* infected root canals did not result in the formation and extrusion of VOCs and chlorinated DBPs.

5.2 Materials and methods

5.2.1 Collection of effluent aliquots

A portable medical suction unit (Armoline, Medical Import Ltd, London, UK) was used during irrigation to aspirate the effluent from the prepared root canals, with the aid of a sterile silicon tube (external diameter: 10mm; Wall Thickness: 2mm) (Ad Fontes Company Ltd, Hong Kong), connected with autoclavable polycarbonate liquid collection jars, assigned for each root specimen separately (Figure 5-1). The use of dental suction was contributory to the simulation of clinical conditions and standards of good endodontic practice.

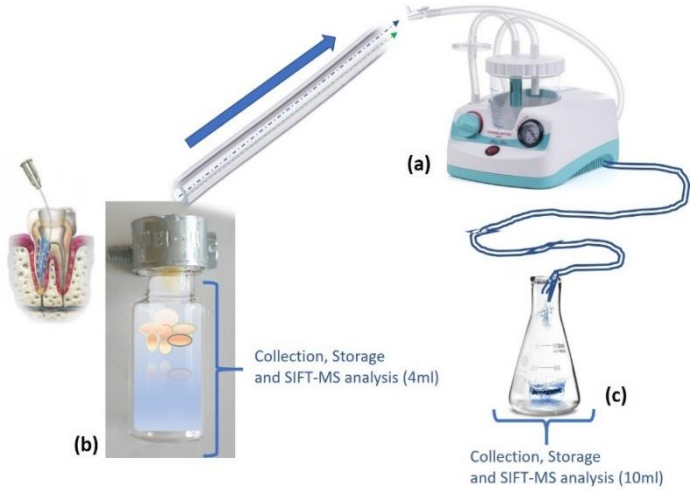


Figure 5-1 Experimental set-up of root canal irrigation and aspiration. (a) Application of portable medical suction. (b) Water-closed apical system filled with sterile ultrapure water. (c) Collection of effluent into polycarbonate jars.

Once chemomechanical procedures were complete as described in Chapter 4, the effluent liquid suspensions obtained from Groups 2 (irrigation with distilled water) and 3 (irrigation with NaOCl and EDTA) were transferred to 20ml sterile Universal tubes and kept refrigerated at -80^{o}C until analysis (Figure 5-1).

5.2.2 Synthesis and characterization of silver-impregnated activated carbon (Ag-AC)

Steam activated and acid washed, highly purified activated carbon (AC) in powder form was obtained as a readily available formulation (Norit ®, Sigma-Aldrich, LOT BCBK2016V). Particle size distribution was measured with a laser diffraction particle-size analyser (CILAS 1180,

Orleans, France). Five samples of AC (10mg) were collected, inserted into a water tank and ultra-sonicated for 30s. The median diameter (D50) of AC particles varied from 22.55μm to 30.36μm.

For the impregnation of AC with silver nanoparticles (AgNPs), an aqueous solution of 0.1M $AgNO_3$ (Sigma Aldrich, UK) was prepared. Amber erlenmeyers were used and 1g of AC was weighted and 20ml of 0.1M $AgNO_3$ was added. A concentrated solution of 2ml NH_4OH (Sigma Aldrich, UK) (estimated concentration of NH_4 at 20^0C= 32% w/v) as a silver (Ag) reducing agent (Tollens reaction) (Acevedo *et al.* 2014). The sample was stirred for 24h, then filtered and washed with distilled water and finally dried at 120°C for 4h.

For the elemental analysis of Ag-impregnated AC (Ag-AC), scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS) (NeoScope JCM-6000plus, Japan) at 10kV was used to confirm the impregnation of AgNPs in AC matrix. The dried Ag-AC powder was attached to 12.5mm aluminium pin stubs (Agar Scientific Elektron Technology, Stansted, UK) using Leit C conducting carbon cement (Agar Scientific Elektron Technology, Stansted, UK) and gold- or carbon- coated for SEM or EDS imaging, respectively.

5.2.3 Antimicrobial efficacy of Ag-AC (broth dilution test)

To study the antimicrobial efficacy of Ag-AC, a direct exposure test was applied against the five microbial species (*E. faecalis strain OMGS 3202, P. acnes, S. epidermidis, A. radicidentis* and *S. mitis*), which were also used for the biofilm development.

To establish the microbial growth, the strains were cultured anaerobically at 37°C for one week on Fastidious Anaerobe Agar (FAA, Lab M, Heywood, UK) supplemented with 5% defibrinated horse blood. Individual starter cultures of each species were collected with inoculation loops (Cole-Palmer, UK), added to phosphate buffered saline (PBS) and incubated anaerobically at 37°C for 3h in anaerobic workstation (MACS-MG-1000, Don Whitley Scientific, UK). The absorbance was adjusted with PBS to 0.5 at 540nm to obtain 10^7cells/ml (Labsystems iEMS Reader MF, Basingstoke, UK).

For each microbial strain, a 2ml suspension was obtained so the final medium contained 10ml. The medium was then treated with 50mg of AC and Ag-AC, for 60min under constant

stirring to ensure direct exposure. The suspensions were then centrifuged to separate the post-treated medium from the powder. Serial dilution of the collected media was performed, followed by dispersion of 0.2ml in FAA plates. Microbial plating was performed in duplicates and the experimental procedures were run in triplicates. The colony-forming units (CFU) were enumerated and expressed logarithmically as mean(SD) log10(CFU) values.

5.2.4 Reduction of available chlorine content of NaOCl solutions after treatment with Ag-AC

Sodium hypochlorite (NaOCl) solutions (1%, 2.5% and 5% v/v) were initially prepared from a stock solution of NaOCl ≥10% v/v (Sigma Aldrich, Gillingham, UK) and their Molarities (M) were verified with a standard iodine/thiosulfate method (iodometric titration) (Chapter 3, Vogel 1962). Then different masses of Ag-AC (5, 10, 50, 100, 200 and 500mg) were added into amber erlenmeyers containing 10ml NaOCl of different concentrations (1%, 2.5% & 5% v/v). The solutions were stirred and homogenized with the aid of a magnetic stirrer for three different time periods of interaction (15, 30 and 60min). From each treated suspension, 9ml aliquots were collected and centrifuged (2000rpm, 22^0C) for 3min. The centrifuged aliquots were sub-divided into three 3ml-containing aliquots. Two ml of each was used, so that iodometric titration was performed in triplicates. Each sample was titrated with a standardized solution of 0.17M sodium thiosulfate ($Na_2S_2O_3$) and the residual chlorine was determined, according to the final stoichiometric measurements:

$C_{NaOCl} \times V_{NaOCl} = C_{Na2S2O3} \times V_{Na2S2O3} / 2.$

5.2.5 Treatment of effluent liquid suspensions with Ag-AC

As previously described in Chapter 4, the infected specimens of Group 1 (n=14; no treatment) were not subjected to any endodontic intervention and the sterile ultrapure water aliquots collected from the periradicular space served as control. The glass vials were then re-filled with sterile ultrapure water and chemomechanical preparation was performed similar to Group 3 (NiTi files and irrigation with 2.5% NaOCl and 17% EDTA). The specimens of previously named Group 1 were now used and re-labelled as Group 4 (Figure 5-2).

The effluent liquid suspensions obtained from Group 4 (n=14) were collected with the aid of the portable medical suction again and were randomly sub-divided into 2 sub-groups (n_a=n_b=7). The specimens of each sub-group were treated with Ag-AC under stirring at 60rpm (Tube Roller, Maple Scientific, UK), at two different mass rates and reaction periods, indicative of full chlorine consumption and no further detectability (results obtained from Section 5.2.4, Chapter 5). After their interaction the carbon-treated effluent aliquots were centrifuged at 2000rpm for 4min and, similarly to Groups 2 and 3, 10ml were collected and transferred to 20ml sterile Universal tubes and kept refrigerated at -80^{o}C until analysis.

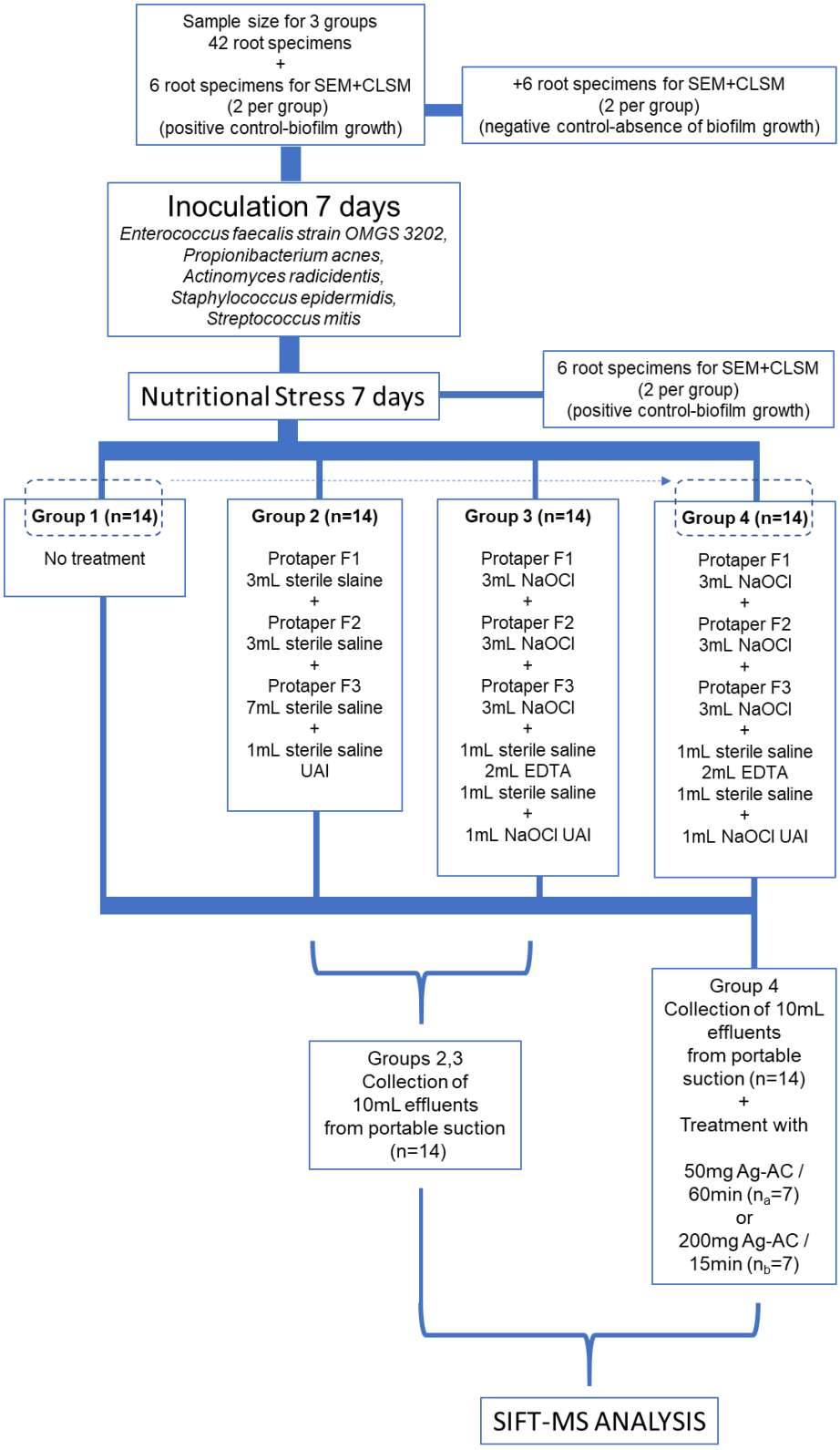


Figure 5-2 Flow-chart of experimental procedures, including sample size calculation, allocation of root specimens, biofilm growth, protocols of root canal chemomechanical preparation and SIFT-MS analysis.

5.2.6 SIFT-MS analysis

For analysis, the samples were defrosted in air. Analysis of the headspace volatile compounds was carried out in real-time by SIFT-MS. The SIFT-MS instrument was operated in two modes: the scan mode where a whole spectrum is captured over a desired m/z range, and the selected ion mode, where individual compounds were targeted and analysed individually. The former is ideal for identifying compounds of interest; the latter enables more accurate quantification. Both modes were used in this study, but quantification was carried out using the selected ion mode. Prior to analysis, three replicate 1ml aliquots of each sample were placed into a sample bag constructed from 50cm length, 65mm diameter Nalophan NA (Kalle, UK), which was then filled with purified air and sealed prior to incubation at 37^{o}C. After equilibrium between the liquid and headspace above it (30min), the headspace was sampled directly into the SIFT-MS via a heated, calibrated capillary that defines the headspace sample flow rate, as is necessary for absolute quantification of VOCs. The analytical downstream quadrupole mass spectrometer was scanned over the range of mass-to-charge ratio, m/z, using the three reagent ions H_3O^+, NO^+ and O_2^+ independently. In addition, individual compounds of interest were selected and the product ions from these compounds were targeted and analysed. From the m/z values of the analyte ions and their count rates, and using the kinetics database stored in the instrument library, the concentrations of the identified VOCs were immediately obtained (Smith & Španěl 2005, Španěl & Smith 2011).

5.2.7 Statistical Analysis

Two-way analysis of variance (ANOVA) with post hoc Tukey tests was used for data analysis of the forming VOCs and DBPs from the effluent solutions as well as the Ag-AC treated effluent solutions. The overall analysis was performed with SPSS software (version 22.0, IBM SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P<0.05$.

5.3 Results

5.3.1 SIFT-MS analysis of effluent aliquots

The headspace of all samples contained several common compounds that are often seen in biological media headspace, including acetone, acetic acid, methanol, ethanol, propanol, acetaldehyde, acetonitrile and ammonia (Table 5-1) (Turner *et al.* 2008, Chapter 4). Sample spectra using the H_3O+, O_2+ reagent ions for Group 3 are presented in Figures 5.3 and 5.4, where analyte ions derived from chloroform and formaldehyde, respectively, can be seen.

Again, the concentrations of all compounds in Group 2 headspace were at a very low ppbv levels. In Group 3 headspace the concentration of all compounds, except for ethanol, presented statistically significant increases compared to Group 2 ($P<0.05$). In Group 3, with the exception of acetone, acetic acid, acetaldehyde and acetonitrile, a large increase in concentration from ppbv to ppmv scales of measurement was noticeable for methanol, propanol, ammonia, chloroform and formaldehyde (Table 5-1).

Table 5-1 Mean (SD) concentrations (ppb.V) of VOCs and DBPs after SIFT-MS analysis of aliquots obtained from effluent aliquots.

Aliquots in effluent (10ml) **Volatile compounds**	**Group 2 Chemomechanical preparation with Distilled Water (n=14)**	**Group 3 Chemomechanical preparation with NaOCl 2.5% and final flush with EDTA 17% (n=14)**
Acetone	11.65 (11.63) [a]	36.52 (23.22) *
Acetic Acid	38.25 (27.12)	139.25 (71.20) *
Methanol	118.87 (69.98)	**3021.19 (986.56) ***
Ethanol	265.53 (87.51)	258.20 (162.79)
Propanol	38.74 (23.82)	**1654.21 (444.06) ***
Acetaldehyde	19.93 (15.98) [b]	**635.56 (132.68) ***
Acetonitrile	4.93 (3.02)	**151.36 (59.55) ***
Ammonia	661.10 (172.13)	**4572.07 (1158.15) ***
Chloroform	62.21 (15.60)	**1852.5 (312.33) ***
Formaldehyde	97.76 (94.61)	**12507.23 (2834.75) ***

[a]: 4 samples presented 0 values; [b]: 6 samples presented 0 values;

*: statistically significant increase in concentration of each volatile compound in Group 3 compared to Group 2 ($P<0.05$);

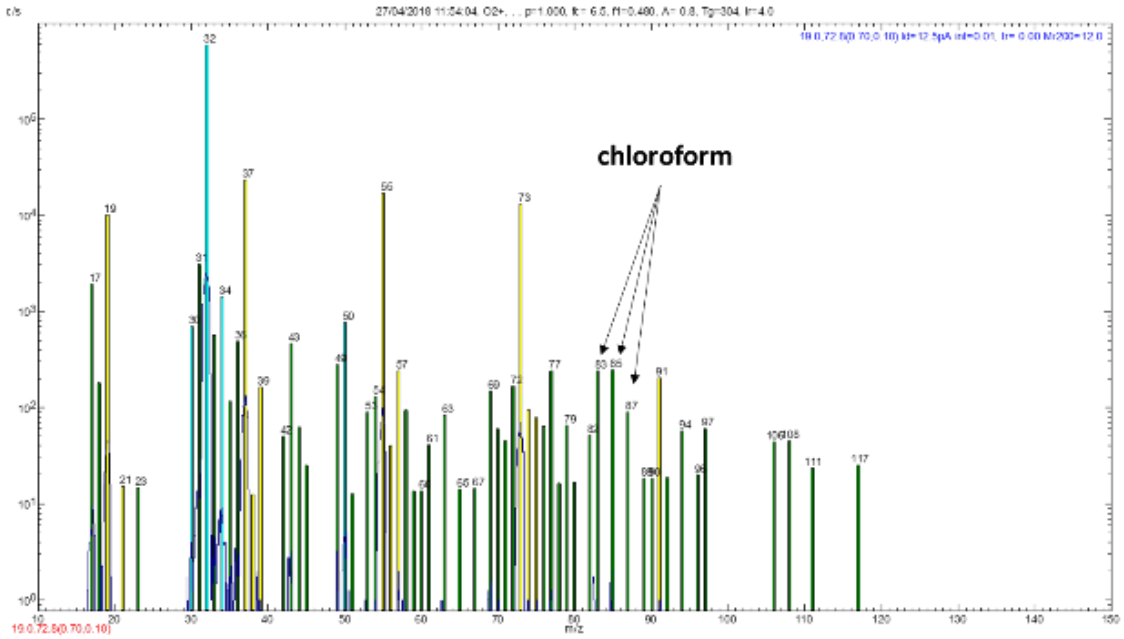


Figure 5-3 O_2+ spectrum of Group 3 aliquot sample (irrigation with 2.5% NaOCl + 17% EDTA) obtained from collected effluent in air. Ions at m/z 83, 85, 87 indicating chloroform is shown on the spectrum.

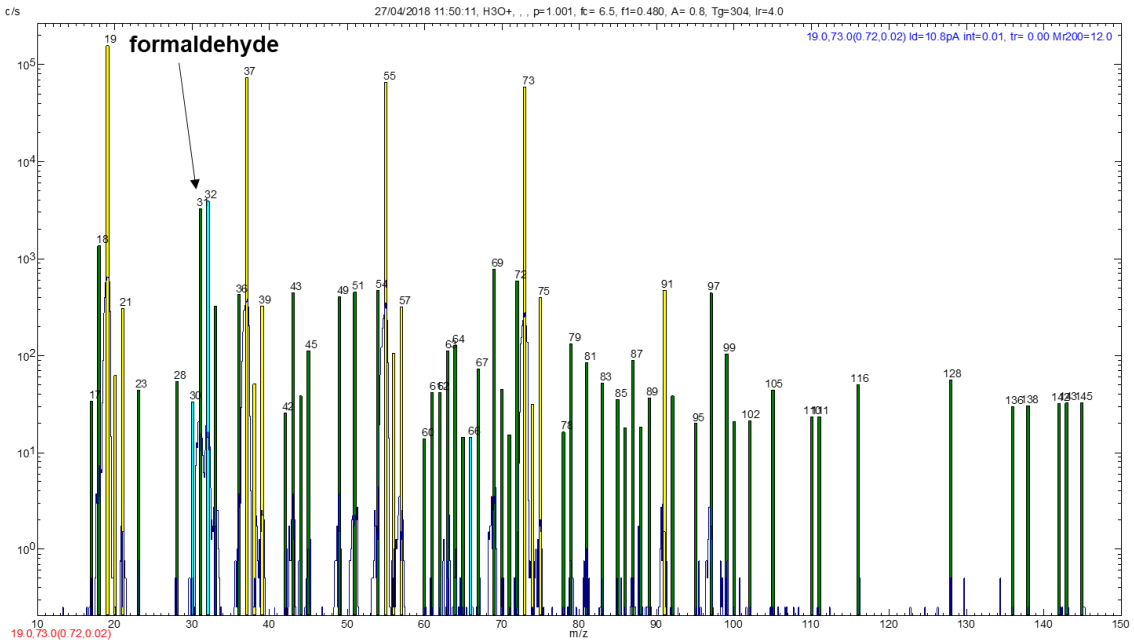


Figure 5-4 H_3O+ spectrum of Group 3 aliquot sample (irrigation with 2.5% NaOCl + 17% EDTA) obtained from collected effluent in air. Ion indicating formaldehyde at m/z 31 is shown on the spectrum.

5.3.2 Characterisation and antimicrobial efficacy of Ag-AC

SEM/EDS analysis confirmed the presence of Ag particles and their impregnation onto the AC mesopore surfaces (Figures 5-5 and 5-6). The use of Ag-AC resulted in statistically significant reduction in microbial counts compared to control and AC in serial dilutions 4 and 5 ($P<0.05$) (Figure 5-7).

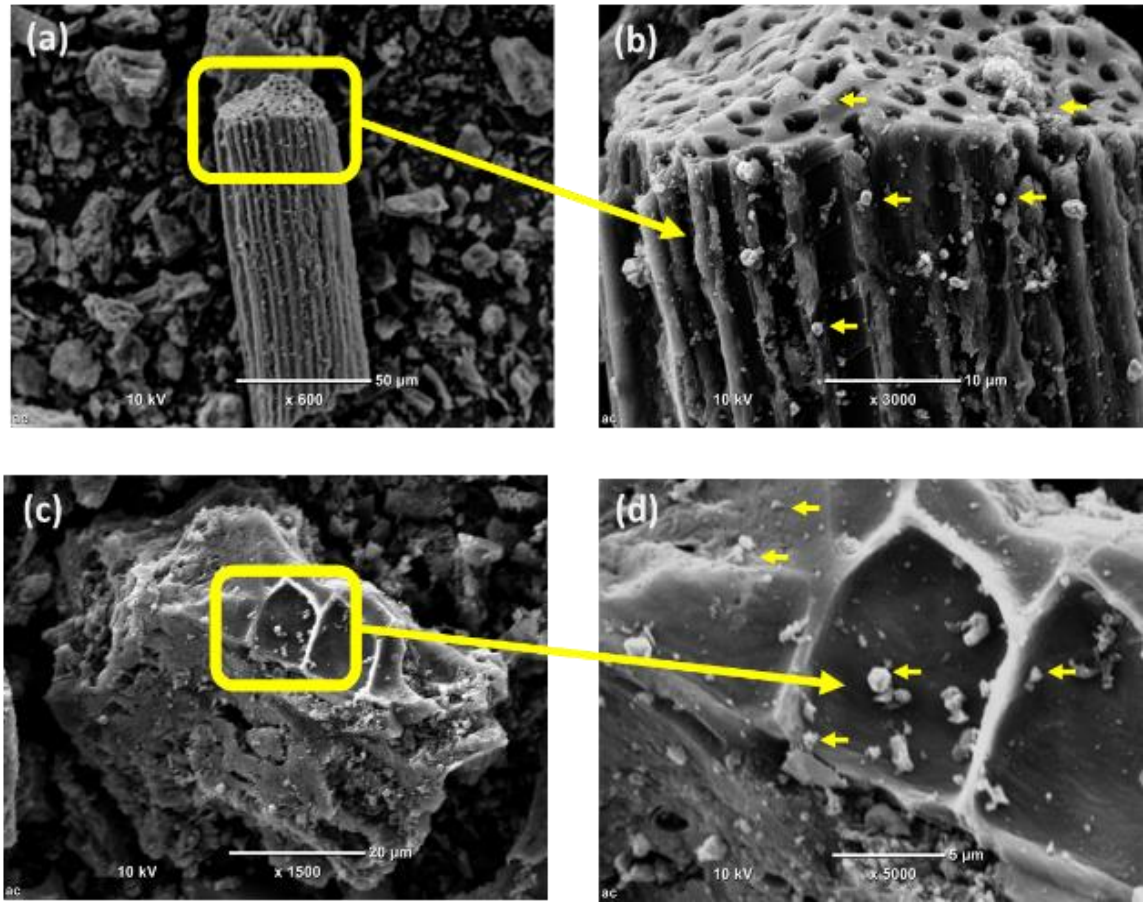


Figure 5-5 SEM images of Ag-AC. (a) Prismatic rod AC particle (50µm scale; x600 magnification). (b) Deposition of Ag particles onto AC crevices (10µm scale; x3000 magnification). (c) Sponge-shaped AC particle (20µm scale; x1500 magnification). (d) Deposition of Ag particles onto AC surface hive (5µm scale; x5000 magnification).

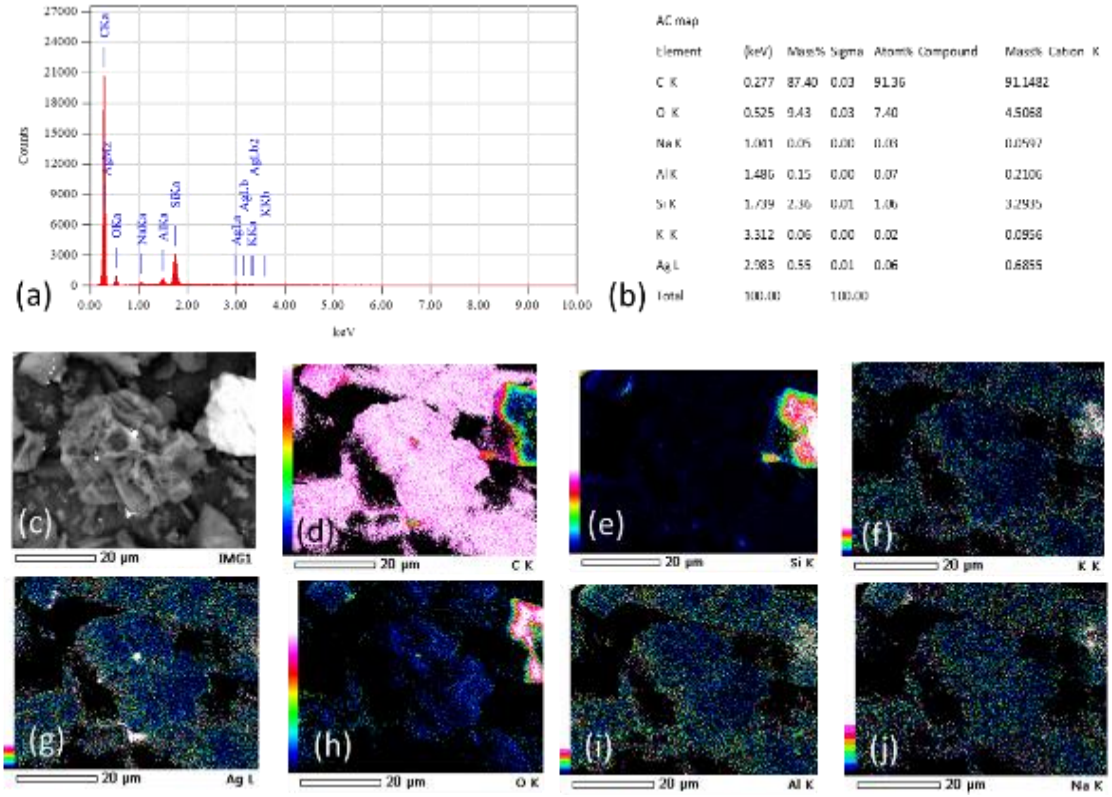


Figure 5-6 EDS analysis of Ag-AC at 20µm scale and x2.2k magnification. (a) EDS spectra. (b) Elemental analysis of the sample obtained by EDS. (c) EDS image. (d-j) Elemental mapping of C(K), Si(K), K(K), Ag(L), O(K), Al(K), Na(K).

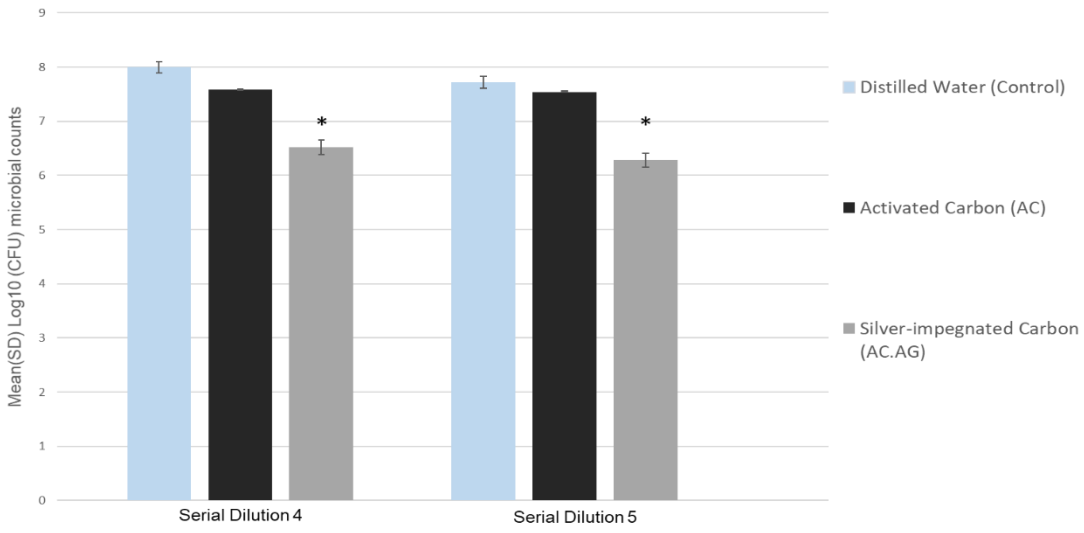


Figure 5-7 Mean(SD) Log10(CFU) reduction in microbial counts following their treatment with AC and Ag-AC, in serial dilutions 4 and 5. *: statistically significant differences compared to rest groups (P<0.05).

5.3.3 Reduction in chlorine content following the interaction of Ag-AC with NaOCl solutions

The interaction of NaOCl with Ag-Ac showed that NaOCl concentration, time of interaction and Ag-AC mass had a significant effect on the available chlorine content ($P<0.05$) (Table 5-2). The minimum time for the complete loss of chlorine ions from NaOCl solutions, regardless of concentration, was 15min, when they interacted with 200mg Ag-AC. The minimum mass of Ag-AC for the complete dechlorination of NaOCl solutions was 50mg, when they interacted for a total period of 60min (Table 5-2). The two aforementioned Ag-AC mass / reaction time ratios were used to determine the effectiveness of Ag-AC in the removal of VOCs and DBPs for a 2.5% NaOCl solution.

Table 5-2 Reduction of chlorine availability following the interaction of 1%, 2.5%, 5% NaOCl with Ag-AC for different time intervals (15, 30, 60 min) and masses (5-500mg).

			Remaining Chlorine Availability (M)						
Irrigant (10mL)	**Molarity (M)**	**Time of interaction (min)**	*Interacting mass of Activated Carbon in powder form (mg)*						
			5	10	20	50	100	200	500
NaOCl 1%	0.1473 (0.01)	15	0.1431 (0.007)	0.1417 (0.005)	0.1318 (0.007)	0.11133 (0.014)	**0.067 + (0.07)**	N/A	N/A
		30	0.1445 (0.004)	0.1431 (0.003)	0.136 (.000)	**0.0822 + (0.007)**	N/A	N/A	N/A
		60	0.1417 (0.003)	0.13 (0.014)	0.119 (.000)	N/A	N/A	N/A	N/A
NaOCl 2.5%	0.36 (0.01)	15	0.3457 (0.003)	0.3414 (0.002)	0.33 (0.003)	0.323 (0.004)	**0.254 + (0.033)**	N/A	N/A
		30	0.33 (0.005)	0.33 (0.007)	0.332 (0.004)	0.296 (0.005)	**0.144 + (0.024)**	N/A	N/A
		60	0.344 (.000)	0.3372 (0.007)	0.319 (0.004)	N/A	N/A	N/A	N/A
NaOCl 5%	0.696 (0.012)	15	0.6928 (0.008)	0.6928 (0.009)	0.6814 (0.005)	0.64 (0.003)	**0.581 + (0.007)**	N/A	N/A
		30	0.6871 (0.007)	0.7026 (0.011)	0.6828 (0.005)	0.6148 (0.002)	**0.5242 + (0.005)**	N/A	N/A
		60	0.6956 (0.005)	0.6715 (0.004)	0.6573 (0.003)	N/A	N/A	N/A	N/A

+: Statistically significant reduction of mean(SD) chlorine concentrations
N/A: non-available

5.3.4 The effect of Ag-AC treatment against VOCs and DBPs in the effluent aliquots

Based on the results obtained from Section 5.3.3 (Chapter 5), the chlorinated effluent aliquots of Group 4 were sub-divided and treated as shown below:

- Group 4a (n=7): Treatment of 10ml effluent with 50mg Ag-AC, for 60min, at room temperature.
- Group 4b (n=7): Treatment of 10ml effluent with 200mg Ag-AC, for 15min, at room temperature.

The treatment of chlorinated effluent aliquots with 200mg Ag-AC for 15min (Group 4b) caused statistically significant reduction in the detectable concentrations of acetone, acetic acid, propanol, acetaldehyde and acetonitrile, compared to Group 4a ($P<0.05$) (Table 5-3). The concentrations of the aforementioned compounds were also significantly reduced compared to the detectable concentrations in Group 3, where Ag-AC was not applied ($P<0.05$) (Table 5-3).

A significant reduction in chloroform concentrations was observed in both Groups 4a and 4b, compared to Group 3 ($P<0.05$). Both protocols of Ag-Ac treatment led to identical residual concentrations of chloroform ($P>0.05$) (Table 5-3).

Ethanol was the only compound that presented a statistically significant increase in concentration in Groups 4a and 4b, compared to Group 3 ($P<0.05$). Methanol, ammonia and formaldehyde remained unaffected by Ag-AC treatments and their concentrations did not statistically differ from those in Group 3 ($P>0.05$) (Table 5-3).

Table 5-3 Mean (SD) concentrations (ppb.V) of VOCs and DBPs after SIFT-MS analysis of aliquots obtained from effluent aliquots.

Aliquots in effluent (10ml) **Volatile compounds**	**Group 2 Chemomechanical preparation with Distilled Water (n=14)**	**Group 3 Chemomechanical preparation with NaOCl 2.5% and final flush with EDTA 17% (n=14)**	**Group 4a Chemomechanical preparation with NaOCl 2.5% and final flush with EDTA 17% + Effluent treatment with Ag-AC 50mg /60min (n=7)**	**Group 4b Chemomechanical preparation with NaOCl 2.5% and final flush with EDTA 17% + Effluent treatment with Ag-AC 200mg /15min (n=7)**
Acetone	11.65 (11.63) [a]	36.52 (23.22) *	22.85 (8.36)	13.13 (9.89) **
Acetic Acid	38.25 (27.12)	139.25 (71.20) *	110.33 (51.92)	41.47 (7.92) **
Methanol	118.87 (69.98)	**3021.19 (986.56) ***	3490.10 (568.99)	3056.58 (614.81)
Ethanol	265.53 (87.51)	258.20 (162.79)	418.03 (231.83) [+]	449.18 (275.18) [+]
Propanol	38.74 (23.82)	**1654.21 (444.06) ***	1042.26 (340.69)	**184.31 (79.96) ****
Acetaldehyde	19.93 (15.98) [b]	**635.56 (132.68) ***	631.43 (78.73)	**362.14 (62.56) ****
Acetonitrile	4.93 (3.02)	**151.36 (59.55) ***	125.71 (11.47)	**82.71 (29.12) ****
Ammonia	661.10 (172.13)	**4572.07 (1158.15) ***	4131.14 (477.64)	4798.43 (1319.27)
Chloroform	62.21 (15.60)	**1852.5 (312.33) ***	244.14 (53.52) **	**193.71 (46.22) ****
Formaldehyde	97.76 (94.61)	**12507.23 (2834.75) ***	14413.88 (2606.11)	11896.18 (2847.95)

[a]: 4 samples presented 0 values; [b]: 6 samples presented 0 values;

*: statistically significant increase in concentration of each volatile compound in Group 3 compared to Group 2 ($P<0.05$);

**: statistically significant decrease in concentration of each volatile compound in Groups 4a, 4b compared to Group 3 ($P<0.05$).

[+]: statistically significant increase in concentration of ethanol of Groups 4a, 4b compared to Group 3 ($P<0.05$).

5.4 Discussion

In this study, the formation of VOCs and DBPS was further documented in the effluent solutions collected, following the simulation of using a dental suction. High concentrations of methanol, propanol, acetonitrile, ammonia, chloroform and formaldehyde were detected in Group 3, following the chemomechanical preparation of the infected root specimens with NaOCl 2.5% and EDTA 17%. In a clinical dental setting, significant volumes of the post-chlorinated effluent solutions may result in waste-water pipelines. To the best of the authors' knowledge, dental units are not supplied with POU treatment or purification systems, as far as chemically derived effluents are concerned. The potential environmental hazards from dental liquid waste have not been determined yet, as their management remains unregulated. The European Union and the WHO have proposed environmental regulations regarding chlorine, chloroform, EDTA and formaldehyde, regarding the quality of water intended for human consumption (EU 1998, WHO 2008). However, the existing concentration thresholds cannot be considered relevant to the measurable concentrations produced in dental effluents.

Based on the results of our preliminary study (Chapter 5.3.2), we proposed the laboratory use of Ag-AC in powder form at two different mass and time ratios, to assess the effect of Ag-AC in chlorine availability with iodometric titration. Dechlorination by AC has been proved to be cost-effective and reliable the removal of residual chlorine in the public water supply industry, since AC acts a reducing agent against chlorine (Jaguaribe *et al.* 2005). The use of Ag-AC effectively reduced the concentration of chloroform, which is in line with current literature findings (Abea *et al.* 2001, Lou *et al.* 2009, Zhao *et al.* 2018). The concentrations of propanol and acetonitrile were also effectively reduced when the aliquots were treated with 200mg Ag-AC for 15min. Therefore, AC has good potential for POU application in the disposal pipes and waterlines of dental units as a filtration system for the adsorption of DBPs, remediation of the post-chlorinated aliquots, as well as removal of residual soluble microbial products (Liu & Li 2015).

On the contrary, the use of Ag-AC did not have any selective adsorptive activity against methanol, ammonia and formaldehyde. Ammonia is one of several forms of nitrogen that exist in aquatic environments. When ammonia is present in water at high enough levels, it is difficult for aquatic organisms to sufficiently excrete the toxicant, leading to toxic buildup in internal tissues

and blood, and potentially death (USEPA 2013). Methanol is significantly less toxic to marine life and many of the effects of short-term exposure are temporary and reversible. Because of its properties, methanol readily mixes with water and evaporates quickly in the atmosphere and would rapidly dissipate into the environment (USEPA 2013). As a VOC, formaldehyde is a natural component of the environment and the human body (SEPA 2018, TURI 2018). It biodegrades readily in air, water and soil under both aerobic and anaerobic conditions. Formaldehyde may be involved in the formation of ground level ozone, which can damage crops and materials (SEPA 2018, TURI 2018). It is not considered possible that formaldehyde pollution has any effects on the global environment, but in the atmosphere, formaldehyde usually breaks down quickly to create carbon monoxide and formic acid, a component of acid rain (Ling *et al.* 2017, Zhu *et al.* 2017). Formaldehyde is highly toxic to aquatic life, such as fish, shellfish and other creatures in rivers, lakes and oceans (Haarstad *et al.* 2012, Lalonde *et al.* 2015), but is not bio-accumulative. Therefore, it is necessary to consider additional POU treatment procedures for the liquid dental waste to reduce or eliminate the accumulated VOCs before their release in the environment.

One parameter that was not documented in this study was the fate of EDTA suspensions. As discussed in Chapter 4, EDTA played a key role in the reaction with NaOCl to form formaldehyde. There is increasing concern about the direct or indirect potential effects of the presence of EDTA in the environment. EDTA may contribute in eutrophication water processes, since the molecule contains approximately 10% of nitrogen that could eventually be available to the aquatic microbiota (Sillanpää 1997). Although the isolated molecule does not present a risk of bioaccumulation, the ligand-metal complexes may significantly increase the bioavailability of extremely dangerous heavy metals that can potentially bind during the flow of the effluent into the waste-water pipelines (Chen & Cutright 2001). In drinking water and waste-water plants, filtering through AC is ineffective to remove the chelate and additional methods such as pre-ozonation or photochemical oxidation systems are required (Rodríguez *et al.* 1999). EDTA may behave as a persistent pollutant in the environment, enhancing the mobility and bioavailability of heavy metals; therefore, there is an urgent need to investigate more on the bioaccumulation of heavy metals in the trophic chain promoted by EDTA and on the remobilization effect of metals in waters and soils (Oviedo & Rodríguez 2003).

Similar to EDTA, the daily use of large volumes of highly concentrated NaOCl solutions may entail environmental implications and risks of toxicity to aquatic organisms. However, considering that NaOCl is extremely reactive, any quantity that flows into the drain from household, dental or industrial use will react with NOM, leading to chlorine loss before it reaches the environment. The additional formation of chlorinated DBPs in effluent requires further examination.

5.5 Conclusion

Within the limitations of this *ex vivo* study, the mechanical preparation and irrigation of artificially infected root canals with rotary NiTi files, 2.5% NaOCl and 17% EDTA resulted in the formation of VOCs and DBPs in aspirated effluent aliquots. The chemical interaction of NaOCl and EDTA resulted in high concentrations of formaldehyde release. The adsorptive capacity of Ag-AC selectively reduced the concentration of chloroform, but had no effect against ammonia, methanol and formaldehyde.

Chapter 6

The synthesis of nano particles of silver-graphene oxide and its efficacy against endodontic biofilms using a novel tooth model

6.1 Introduction

Bacterial growth within a necrotic root canal is the main cause of apical periodontitis (Kakehashi *et al.* 1965, Möller *et al.* 1981). Periradicular tissue healing is relevant to the accomplishment of adequate elimination or reduction of bacterial population (Byström *&* Sundqvist 1981, Dalton *et al.* 1998). However, cleaning and debridement still remain challenging issues because the bacteria in root canals adapt within stressed micro-environmental conditions usually in the form of a biofilm (Costerton *et al.* 2003, Donlan 2002). The properties of bacteria that inhabit a biofilm are different from those of planktonic cells, because the bacterial congregations possess a higher resistance towards antimicrobial agents (Costerton *et al.* 1999).

Instrumentation allows for removal of majority of the root canal content within the main root canal space. However, experiments on extracted teeth have shown that, during preparation, up to 48% of canal walls are left un-instrumented (Peters *et al.* 2001, Peters *et al.* 2010) therefore large areas of biofilm along with necrotic tissue remnants may potentially be left behind. The formation of biofilm in the accessory root canal system, such as in lateral canals that communicate with surrounding bone, is particularly problematic since these areas are difficult to debride (Chapter 1; Figure 1-2). Persistent infection in such confined areas is regarded as a common cause of root canal treatment failure and post-treatment apical periodontitis (Love 2001, Nair 2004, Ricucci *et al.* 2013).

The root canal has a complex anatomy and for the antimicrobial irrigant to be effective it is important that it reaches the biofilm in the entire canal to disrupt it completely (Niazi *et al.* 2014). Furthermore, irrigant agitation and activation techniques may be necessary to aid the dispersal of the irrigant into inaccessible areas (Macedo *et al.* 2014) and minimise the negative effect of irrigant stagnation and apical vapor lock within confinements of a close root canal system (Mohhmed *et al.* 2016). Multiple activation methods have been proposed to improve the efficacy of irrigants, including sonic, ultrasonic, negative apical pressure irrigation, as well as laser

activation (Gu *et al.* 2009). Ultrasonically activated irrigation relies on the transmission of acoustic energy from an oscillating file with no active tips or a smooth wire to an irrigant in the root canal. The energy is transmitted by means of ultrasonic waves and can induce acoustic streaming and cavitation of the irrigant (van der Sluis *et al.* 2007). XP Endo Finisher (XPEF) (FKG Dentaire, Switzerland) has been recently introduced as a final disinfection step to disturb the bacterial biofilm, which is a 25/.00 instrument produced using a special type of alloy, the NiTi MaxWire (Martensite-Austenite Electropolish-FleX, FKG) (Trope *&* Debelian 2015). According to the manufacturer, the file is straight in its M phase when it is cooled, and changes into A phase when it is exposed to body temperature where it acquires its unique spoon shape with a length of 10 mm from the tip and a depth of 1.5 mm because of its molecular memory (Chapter 1; Figure 1-12) (Trope *&* Debelian 2015, FKG Dentaire SA 2016).

In order to understand the flow of irrigants in root canals, computational fluid dynamics in transparent root models have been used to measure physical parameters, associated with irrigant flow in the main and lateral canals (Boutsioukis *et al.* 2010, Robinson *et al.* 2015, Wang *et al.* 2015). Other studies have attempted examination of irrigant flow and antimicrobial effectiveness in less accessible areas of the root canal system, such as lateral canals using in 3-D printed or solidified polydimethylsiloxane models (Macedo *et al.* 2014, Mohmmed *et al.* 2017). However, limited information exists regarding the antimicrobial effectiveness of irrigation and activation protocols in the main and lateral canals, within human tooth specimens. Hence to be able to more closely, simulate clinically relevant conditions it is vital to develop an infected tooth model that can subjected to fluid dynamic principles.

Sodium hypochlorite (NaOCl) (1-5%) is currently regarded as the gold standard for irrigation and chemical disinfection of the root canal system (Sedgley 2004). This is due to its antimicrobial effects (Davis *et al.* 2007, Siqueira *et al.* 2000), its ability to disintegrate and solubilize organic tissue (Clarkson *et al.* 2006, Combakara *et al.* 2010) and to denature toxins (Buttler *&* Crawford 1982). However, NaOCl is known for its deleterious effects and caustic effects against dentine collagen, soft tissues and cancellous (Oyarzún *et al.* 2002, Pashley *et al.* 1985, Kerbl *et al.* 2012). In addition, a recent study and the results presented in the previous Chapters in this thesis reported the formation of toxic volatile compounds and chlorinated disinfection by-products when it reacts with compounds of infected root canal content (Varise *et al.* 2014). Chlorhexidine (CHX)

(0.2-2%) also has a broad-spectrum antibacterial action (Ercan *et al.* 2004, Siqueira *et al.* 2007, Zamany *et al.* 2003) and its use as a final irrigant has been recognised as advantageous, due to its substantive effect on dentine walls (Khademi *et al.* 2006, Leonardo *et al.* 1999), however, it lacks soft tissue dissolution capacity (Naenni *et al.* 2004). The use of a demineralizing agent, such as EDTA (17%), has been indicated for removal of contaminated inorganic content of the smear layer, which is deposited on dentine walls during instrumentation (Patterson 1963). However, the chelating action of EDTA has been associated with loss of inorganics, reduction in dentine microhardness and erosion of dentine tubules when combined with NaOCl (Zhang *et al.* 2010). The capacity of EDTA to disrupt biofilms also remains questionable (Yoshida *et al.*1995).

The current status of endodontic irrigants, raise issue with regards to biocompatibility, biofilm resistance within complex root canal anatomy and restrictions related toy factors such as concentration, time and volume, makes it is important to develop new endodontic biomaterials for promotion of root canal disinfection. In addition, antibiotic resistance and prevalence of biofilms are major threats, which has triggered the exploration of alternative approaches to minimise the burden (Stewart & Costerton 2001, Bassegoda *et al.* 2018). The incorporation of NPs to advocate novel disinfection strategies has gained attention in the past few decades due to their innovative and functional properties. The bactericidal effects of metallic NPs that stem from their biophysical properties is of huge interest and the potential of nano- silver, zinc oxide, titanium dioxide and GO are being explored for different biomedical applications (Allaker & Memarzadeh 2014, Guo *et al.* 2017).

Carbon materials are known to be more environmentally and biologically friendly than other inorganic materials. GO is chemically exfoliated from oxidized graphite and constitutes of monolayer of carbon nanosheets that form dense honeycomb structures (Novoselov *et al.* 2004, Geim & Novoselov 2007). These contain hydroxyl and epoxide functional groups on the two sides and carboxylic groups at the edges (Li *et al.* 2008, Park & Ruoff 2009). GO has been reported to exhibit antibacterial activity against several bacterial species (Akhavan & Ghanderi 2010, Tu *et al.* 2013, He *et al.* 2015, Nanda *et al.* 2016) and is considered as a promising material for biological applications (Chung *et al.* 2013). On the hand, silver nanoparticles (AgNPs) have been reported to promote enhanced antibacterial activity compared to conventional silver-based materials (Sondi & Salopek Sondi 2004, Sharma *et al.* 2009, Rai *et al.* 2009, Choi *et al.* 2008, Choi & Hu

2008, Siddiqi *et al.* 2018). Recent studies have shown that a hybrid biomaterial of AgNPs, where a layered material like GO is used as matrix, can induce binding capability, that usually lacks in AgNPs alone and thus is able to enhance antimicrobial action (Das *et al.* 2011, Song *et al.* 2016).

This study reports the synthesis of Ag NPs on GO particles (Ag-GO) with the antimicrobial and biofilm disruption ability of Ag-GO particles compared to sterile saline, EDTA 17%, CHX 2%, NaOCl 1% and 2.5%, with conventional irrigation (CI), ultrasonically activated irrigation (UAI) and XP Endo Finisher (XPEF). A newly proposed infected tooth model was used to validate the *in vitro* findings.

6.2 Materials and methods

6.2.1 Specimen selection and preparation

Freshy extracted single rooted teeth with a single canal, free of cracks, fractures, caries, abrasions and discolouration were collected, in accordance with the protocol outlined in the Research Ethical Committee Document (Wales REC 4, 14/WA/1004, UK). For a number of 20 groups, total sample size was calculated 120 (n=6 per group) with G*Power 3.1.9.2 software (Franz Faul, Universitaet Kiel, Germany) with an a-value of .05, an actual power of 80% and an effect size (f) of 0.23 (partial η^2=0.05).

All specimens were initially autoclaved at 121°C for 15min. The crowns of teeth were removed and the root length was standardised to 15mm. Root canals were accessed and scouted with K-files size 10 (Dentsply Sirona, Ballaigues, Switzerland) to ensure patency until their tip was detected through apical foramen, under magnification with an operating dental microscope (Global, USA). The working length was established by subtracting 0.5 mm. The root canals were prepared with ProTaper Universal (Dentsply Sirona, Switzerland) rotary files up to F3 instrument (apical diameter 0.30 mm, taper 0.09). Irrigation was performed with NaOCl 5.25% (Chloraxid, Cerkamed, PL) using a total volume of 8ml (2ml per file sequence). EDTA 17% (Schottlander & Davis, UK) (2ml) was used to remove the inorganic phase of the smear layer. Root canals were finally flushed with 2ml distilled water and dried with sterile paper points.

Each tooth was longitudinally hemi sectioned in a bucco-labial orientation with a wafering blade (Niazi *et al.* 2014). The specimen half with the most conserved root canal was used for the preparation of two artificial lateral canals using a cylindrical diamond bur with 0.25mm radius (111-010M pk5, Dentsply, Switzerland), under magnification with an operating dental microscope (Global, USA). The lateral canals were prepared in the apical (3mm from root apex) (LAT1) and middle third (6mm from root apex) (LAT2). These specimens were selected for the biofilm growth.

6.2.2 Artificial lateral canal characterisation

To ensure standardisation in the creation of artificial lateral canals and thus homogeneity in lateral canal dimensions in all samples entered into the study, 7% of the samples (n=8) were randomly selected and digitally scanned using non-contacting laser profilometry (NCLP) (XYRIS 4000CL, Taicaan Technologies, UK). The NCLP had a 2μm spot size, with dimensional accuracy 10nm (z), <1um (x, y) and reproducibility accuracy 238nm. Lateral canals were scanned with scanning x (width), y (length) dimension 3.5x1.5mm using a 10um step over, producing digital point-cloud data of 53,001 individually measured points per lateral canal (Mylonas *et al.* 2018).

Laterals canals were then dimensionally analysed for their mean x, y dimensions (mm) using automated in-software digital callipers, whilst mean z (depth) dimension (mm) was measured using 3-D step height according to ISO 5436-1 (Mylonas *et al.* 2018). Dimensional analysis was calculated using a bespoke automated macro written to allow objective analysis of all digital data sets using specialist metrology software (MountainsMap, Digital Surf, France). The process of lateral canal scanning, digitisation, and lateral canal dimensional analysis is summarised in Figure 6-1.

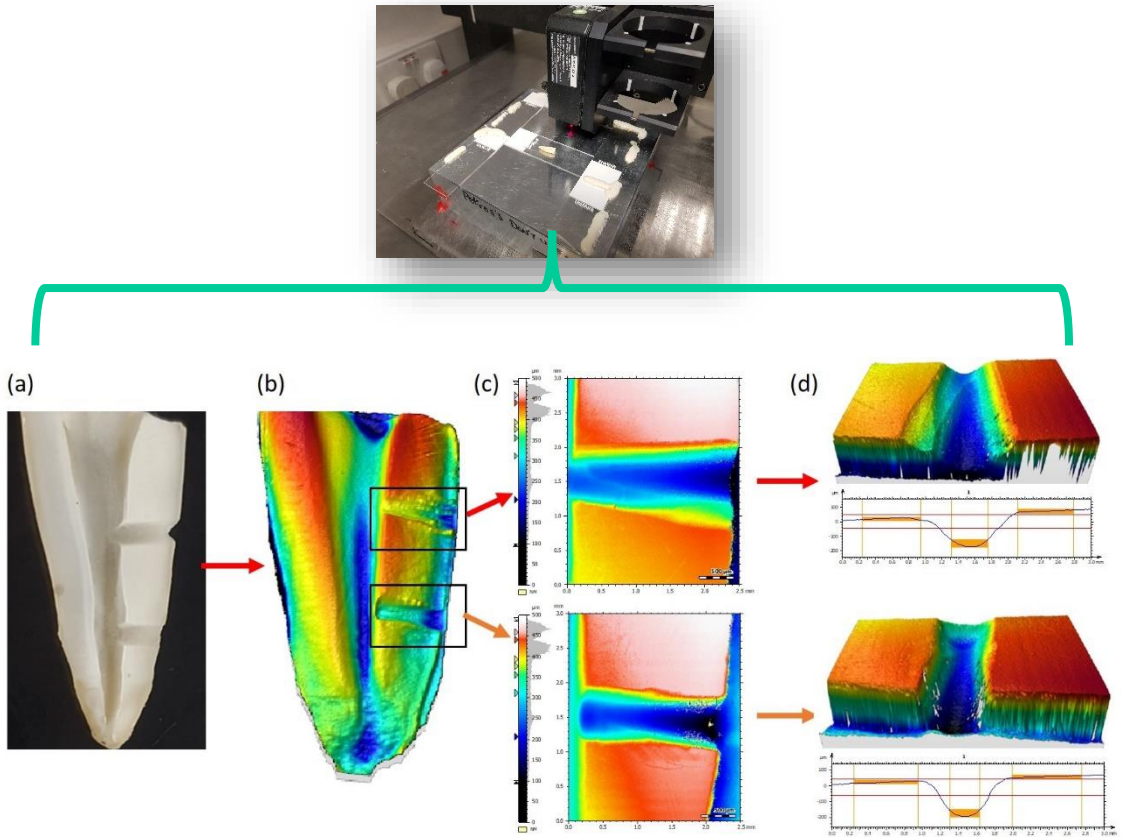


Figure 6-1 Artificial lateral canal characterisation. (a) Hemisected tooth model specimen. (b) Tooth model digitised using non-contacting laser profilometry. (c) Lateral canal dimensional analysis. (d) Automated dimensional analysis, x,y dimensions using digital calipers and z dimension using 3D step height analysis according to ISO 5436-1. The relevant data were expressed as mean (SD) 3D step height (mm).

6.2.3 Fabrication of testing apparatus

A novel testing apparatus was fabricated to simulate the conditions of intracanal irrigation, with the use of an *ex vivo* closed apical system, to reflect periapical tissue resistance to irrigant extrusion (Psimma *et al.* 2013). Prior to hemi sectioning, each root specimen was vertically stabilised on its coronal surface with thermo-plasticised silicone glue. A zinc plated mini fuel hose line pipe clip (diameter: 11-13mm, height: 10mm) (Wilson Lendrum & Weir, UK) was positioned in the periphery of the root specimen to achieve a centring position of specimen within the diameter of the clamp margins. The clamp was then filled with injectable thermo-plasticised silicone glue with the aid of a glue gun (Bosch PKP 18 E, Robert Bosch Holdings, UK) (Figure 6-2 a). The root specimen was covered up to apical third by silicone glue which was cylindrically shaped after application of vertical pressure with the aid of a 3-ml clear glass bottle (Ampulla,

Cheshire, UK) (Figure 6-2 b). A polyvinylsiloxane silicone (Aquasil, Dentsply DeTrey, Konstanz, Germany) key impression was taken for the apical root segment and the cylindrical silicone index to ensure apical seal and to simulate apical tissue resistance (Figure 6-2 c).

After the hemi section of the specimens, the split halves (Figure 6-2 d & e) were re-approximated within the apparatus and further merged into the apical silicone index. The clamp was tightened with the aid of an electric screwdriver (Bosch IXO, Robert Bosch Holdings, UK), to ensure the performance of the apparatus as a single unit (Figure 6.2 f-h). Prior to use, every surface of the testing apparatus elements was sterilised under UV irradiation for 2h.

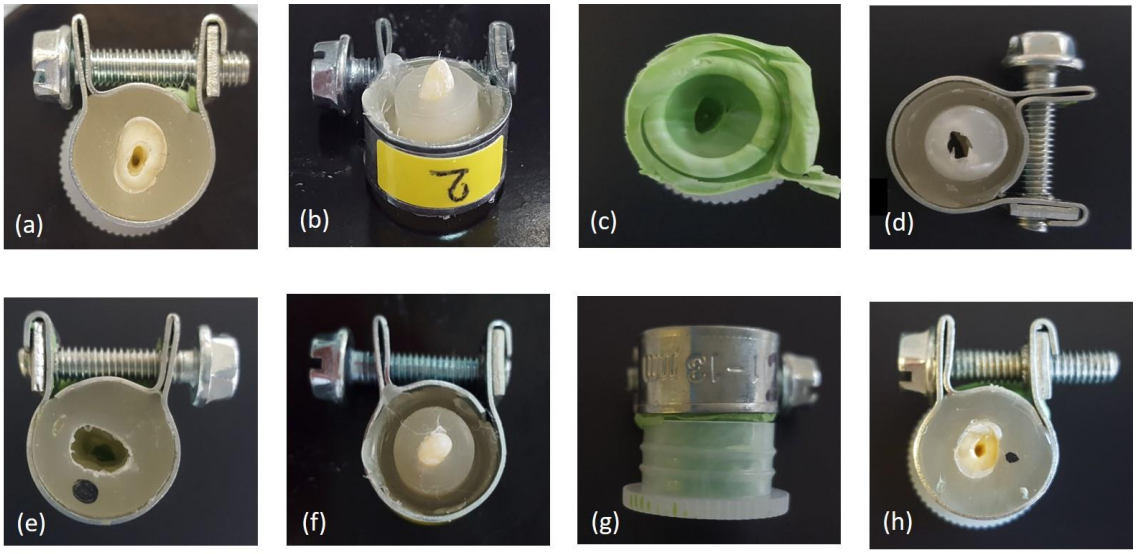


Figure 6-2 Infected tooth-model testing apparatus. (a) Coronal view of clamp-silicon index. (b) Apical view of clamp-silicon index. (c) Silicon key-impression of apical root segment. (d, e) Removal of root specimen and inspection of silicon internal surfaces to ensure absence of structural deficiencies after setting. (f, g, h) Re-assembling of hemisected root specimens, addition of silicon key to simulate apical pressure resistance and tightening of the clamp.

6.2.4 Development of multispecies stressed biofilm in hemi-sectioned root halves

One hundred and fourteen pairs of root halves (n=114) were randomly assigned to 1 positive control (biofilm growth/no treatment) and 18 treatment groups. Each of the 19 groups included six pairs of root halves. Six additional pairs of autoclaved, sterile root halves served as negative control group (no biofilm growth).

A stressed multispecies biofilm comprising of five selected bacteria was developed on the root canal of each selected hemi section using the protocol developed by Niazi *et al.* (2014). The selected endodontic bacteria in this biofilm included *Propionibacterium acnes*, *Actinomyces*

radicidentis, Staphylococcus epidermidis, Streptococcus mitis -recovered from root canals of teeth with refractory endodontic infections- and *Enterococcus faecalis* strain OMGS 3202 (Dahlén *et al.* 2010, Niazi *et al.* 2010).

To establish biofilms, the strains were cultured anaerobically at 37°C for seven days on Fastidious Anaerobe Agar (FAA) supplemented with 5% defibrinated horse blood (FAA, Thermo Scientific™, UK) (Figure 6-3), suspended in modified fluid universal medium (mFUM) (Gmür *&* Guggenheim 1983) and incubated at 37°C for 3h in anaerobic workstation (MACS-MG-1000, Don Whitley Scientific Ltd, UK). The absorbance was adjusted to 0.5 at 540nm to obtain 10^7 cells/ml. (Labsystems iEMS Reader MF, Basingstoke, UK).

The selected root halves were autoclaved at 121°C for 15min, placed in 2ml of mFUM and pre-reduced in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% carbon dioxide) for 2h. Each root half was further seeded with 400µl of each of the five starter cultures. The biofilms were grown anaerobically with regular medium change after every 24 h for the first 7 days and the biofilms were left to grow for the next 7 days in the unchanged medium in order to stress the microorganisms nutritionally (Niazi *et al.* 2014) (Figure 6-4).

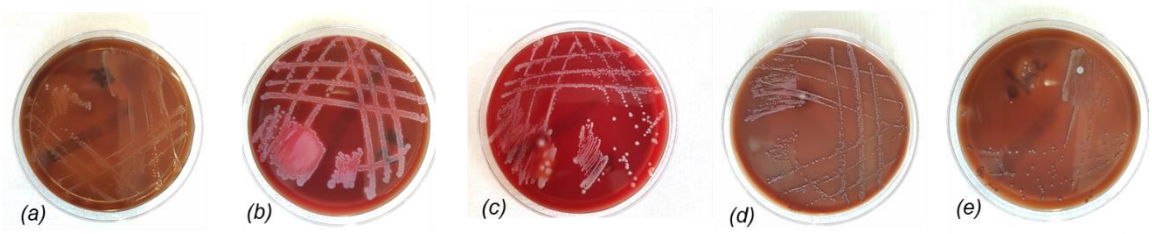


Figure 6-3 Anaerobic cultures of microbial strains at 37°C after seven days on Fastidious Anaerobe Agar supplemented with 5% defibrinated horse blood. (a) *E. faecalis OMGS 3202.* (b) *S. epidermitis.* (c) *P. acnes.* (d) *A. radicidentis.* (e) *S. mitis.*

Figure 6-4 Macroscopic appearance of biofilm growth on sterile human dentine root specimens.

6.2.5 Synthesis and characterisation of Ag NPs in GO aqueous solution (Ag-GO)

A GO aqueous dispersion (10mg/ml) (GoGraphene, William-Blythe, Harlow, UK) was used and the dispersion was progressively diluted in sterile distilled water to a lower concentration below 6mg/ml to ensure transition from pseudoelastic fluid to Newton fluid state (Shu *et al.* 2016). To ensure effective syringe delivery with the aid of open-ended irrigation needle (27-G), the solution was finally diluted to 2.5mg/ml.

Ag NPs were prepared by reducing $AgNO_3$ with sodium borohydrate ($NaBH_4$) in presence of GO suspension, according to the method by Das *et al.* (2011). Typically, 10ml of homogeneous suspension of GO (2.5mg/ml) was mixed with 10ml aqueous $AgNO_3$ solution 0.01M (Sigma-Aldrich, Gillingham, UK) and the reaction mixture was stirred for 30min at room temperature, before the addition of the reducing agent. Subsequently, 1ml of a freshly prepared solution of $NaBH_4$ 0.01M (Sigma-Aldrich, Gillingham, UK) was added slowly to the reaction mixture of $AgNO_3$–GO suspension with vigorous stirring. The colour of the reaction mixture turned into dark brown to grey. The reaction mixture was stirred for another 5h for complete reduction at room temperature.

For elemental analysis of Ag-GO, scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS) (NeoScope JCM-6000plus, Japan) at 10kV was used to confirm the impregnation of Ag NPs in GO matrix. One millilitre (1ml) of Ag-GO suspension was left to air-dry and the solid sheets were placed on 12.5mm aluminium pin stubs (Agar Scientific Elektron Technology UK, Stansted, UK) using Leit C conducting carbon cement (Agar Scientific Elektron Technology UK, Stansted, UK) and gold- or carbon- coated for SEM or EDS imaging, respectively. The sizes and morphologies of GO and Ag-GO were characterized by scanning transmission electron microscopy (STEM) (JEOL JEM-F200 microscope, Japan) at 200kV.

6.2.6 Protocols of irrigation procedures

The irrigants tested in experimental groups were: Sterile saline (NaCl 0.9%) (JFA Medical, Blackpool, UK), EDTA 17% (Schottlander & Davis, UK) NaOCl 1%, NaOCl 2.5%, chlorhexidine 2% (CHX) (GlucoChex, Cerkamed, PL) and Ag-GO (2.5mg/ml). Concentrated NaOCl solutions were prepared from a stock solution NaOCl ≥10% (Sigma Aldrich, Gillingham, UK) and verified with iodometric titration (Vogel 1962). After NaOCl irrigation, the root canals were washed with 1ml sodium thiosulphate (Na_2SO_4) 0.1M (Sigma Aldrich, Gillingham, UK) to neutralise any residual chlorine activity. Three types of irrigation procedures were used: Conventional irrigation (CI), ultrasonically activated irrigation (UAI) and irrigation with application of XP Endo Finisher (XPEF). The performance of the respective irrigation and agitation procedures is summarised in Figure 6-5 (a-c).

Syringe irrigation was performed using a 27-G open-ended needle and a 3ml-containing syringe with a luer-lock (Monoject, Medtronic, UK). A rubber-stop was applied 3mm short of working length. Irrigation was carried out by an accredited Specialist Endodontist using digital pressure with the forefinger only at a rate compatible with the time stated for each irrigation procedure. The needle was moved up and down in the canal gently, ensuring that it did not bind on axial walls.

Ultrasonically activated irrigation was conducted with a size 25 ultrasonic file with zero taper (Irrisafe, Acteon, UK) placed in the canal, filled with each irrigant type. The ultrasonic unit (Newtron Booster, Acteon, UK) was adjusted for endodontic use (ring colour at yellow) with a power setting at 9, according to the manufacturer's recommendations. The file was inserted into the canal 1mm short of working length. File oscillation was performed towards the lateral canals' direction with no constraint.

The XP Endo Finisher file was inserted vertically in gentle longitudinal vertical motion of 7-8mm against the sidewalls of the canals. The insertion depth was 1mm short of the working length and it was operated according to the manufacturer's instructions, with a motor rate of 800rpm and torque 1Ncm. To ensure file efficacy, each allocated tooth specimen was treated immediately after removal from 37^{o}C anaerobic incubation.

6.2.7 Determination of quantitative viable counts of the biofilms after irrigation

Once irrigation procedures were complete, the clamp of the silicone-fused hose was untightened, the apical silicone index was removed, the merged root fragments were simultaneously taken out of the apparatus and the chosen root half with the two lateral canals was placed in a 9-cm sterile Petri dish (SLS, Nottingham, UK). For the quantitative measurement of the microbial viable counts, 3-point sampling (MAIN: entire canal; LAT1: middle third lateral canal; LAT2: apical third lateral canal) was performed using sterile paper points (Protaper Universal F3 Paper point, Dentsply Sirona, Switzerland) (Fig. 6-5 d). Sampling in the main canal was performed by soaking a paper point within the length of the canal lumen for 30s. Sampling in the lateral canals was performed by inserting the paper point tips only within their dimensions for 30s.

The sampled paper points were dispersed into 1ml of BHI (Brain-Heart infusion Broth, Lab M) and vortexed for 2min. After serial dilution in BHI, aliquots (100µl) were plated onto duplicate FAA plates and incubated anaerobically at 37°C for 7 days. After the incubation period, the numbers of colonies and their $\log_{10}$ ($\log_{10}$CFU) were counted.

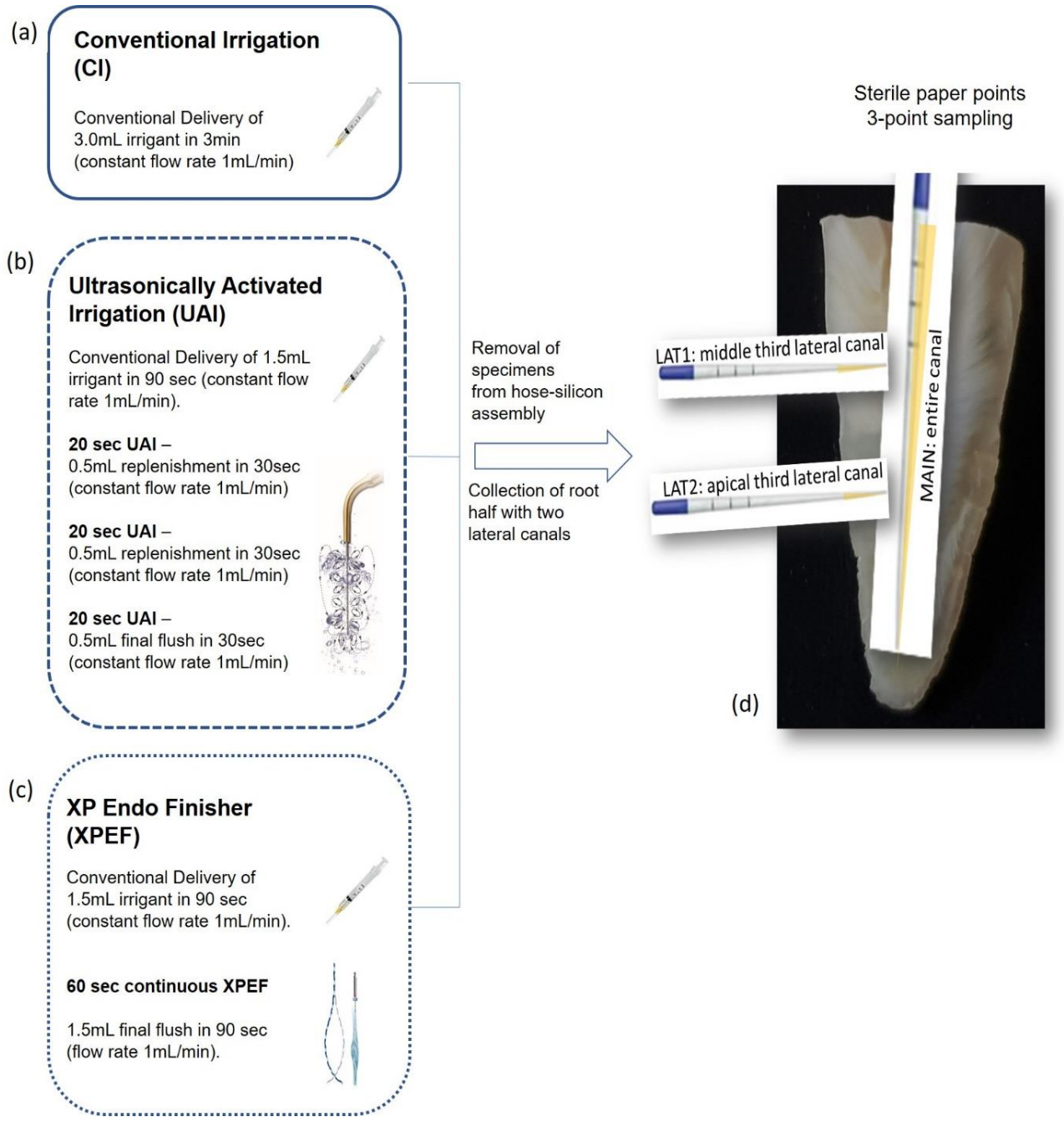


Figure 6-5 Protocols of irrigation procedures and 3-point sampling areas. (a) Conventional irrigation (CI). (b) Ultrasonically Activated Irrigation (UAI). (c) Application of Endo XP Endo Finisher (XPEF). (d) 3-point paper point sampling areas including main canal lumen (MAIN), middle root third lateral canal (LAT1) and apical root third lateral canal (LAT2).

6.2.8 Confocal Laser Scanning Microscopy (CLSM) analysis of the multispecies biofilm

After sampling for quantitative viable counts, the biofilms of 3 specimens per group were stained with a Live/Dead Baclight bacterial viability kit (Thermofisher Scientific, UK) and visualized under a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Milton Keynes, UK). A x63 magnification oil immersion objective with a numerical aperture of 1.40 and a confocal pinhole to Airy 1 unit was used to observe the fluorescence emission of SYTO® 9 and Propidium Iodide using 488 nm and 569 nm (Ar-Kr laser) as the excitation source, respectively.

Image acquisition was performed with a zoom factor of 4.0, a pixel resolution of 0.11 µm/pixel and field resolution of 512 x 512 pixels. Three z-stacks were acquired from each root specimens: apical third of the main canal, middle lateral canal and apical lateral canal. Each stack had a substratum coverage field area of 59.52µm x 59.52µm. For each stack, the z-step for the images was 2µm and 10 2-D images were acquired. The acquired images of all biofilms in each group were analysed using bioImage_L (Chávez de Paz 2009). Biofilm disruption was expressed by mean [standard error(se)] values of total biovolumes (um^3/um^2) and mean percentages (%) of dead (red), live (green) and unknown (orange) populations within residual biofilms.

6.2.9 Statistical Analysis

Two-way analysis of variance (ANOVA) with post hoc Tukey tests was used for data analysis and comparison of the quantitative viable counts amongst all groups. The overall analysis was performed with SPSS software (version 22.0, IBM SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at $P<0.05$. The acquisition of total biovolume (um^3/um^2) and percentage of biofilm viability (% green/red/unknown) for each group was performed with BioImage_L (Chavez de Paz 2009), by performing two-way analysis of variance (ANOVA).

6.3 Results

6.3.1 Artificial lateral canal characterisation

Dimensional analysis revealed that the artificial middle lateral canals mean (SD) x, y, z dimensions were 0.383(0.057) mm, 2.232(0.213) mm and 0.256(0.044) mm, whilst the apical lateral canals mean (SD) x, y, z dimensions were 0.371(0.061) mm, 1.737(0.204) mm, 0.216(0.049) mm. No statistically significant difference (P>0.05) was observed in the dimensions between either type of lateral canal (Table 6-1).

Table 6-1 Mean (SD) length (y), width (x) and depth (z) (mm) are shown for both middle and apical artificial lateral canal.

Lateral Canal Dimensional Analysis		
	Middle (LAT1) (n=8)	Apical (LAT2) (n=8)
Mean (SD) Length (y) (mm)	2.232(0.213)	1.737(0.204)
Mean (SD) Width (x) (mm)	0.383(0.057)	0.371(0.061)
Mean (SD) Depth (z) (mm)	0.256(0.044)	0.216(0.049)

6.3.2 Characterisation of Ag-GO with SEM/EDS and TEM

SEM/EDS analysis confirmed the presence of Ag and its impregnation within the GO structure (Figure 6-6 a-c). In addition, areas of Ag agglomeration were noticeable with diameters varying from 7 to 20μm (Figure 6-6 a). The analysis of TEM images confirmed the morphology of polygonal GO sheets (Figure 6-7 a-c). Ag-GO dispersion contained spherical Ag NPs of diameter 20-50nm, which were evenly distributed and anchored, forming a network on the surface of GO sheets (Figure 6-8).

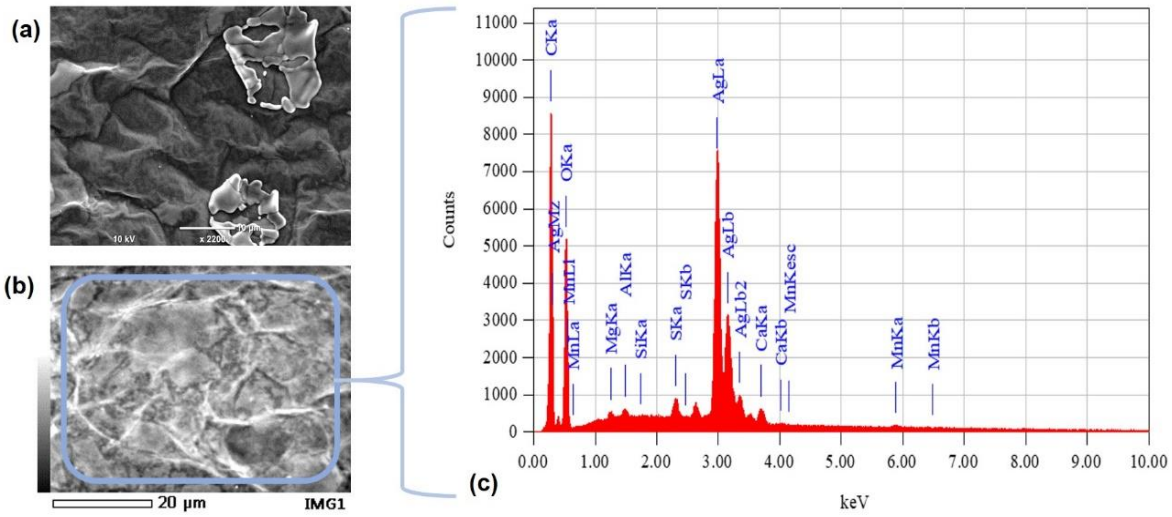


Figure 6-6 (a) SEM analysis of Ag-GO precipitates in aqueous solution at 10µm scale and x2.2k magnification. Silver agglomeration is present. (b, c) Elemental analysis of the sample obtained by EDS.

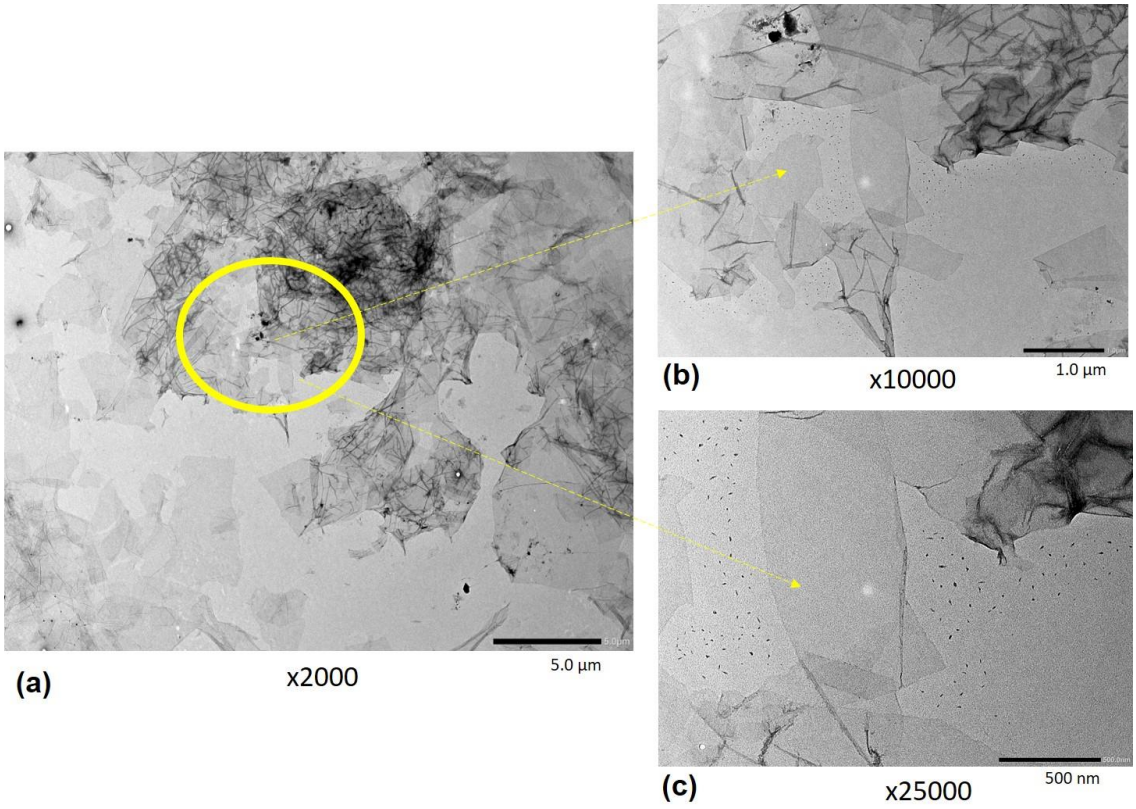


Figure 6-7 TEM images of graphene oxide at different magnifications and scale bars (a; x2000 / 5µm, b; x10000 / 1µm, c; x25000 / 500nm).

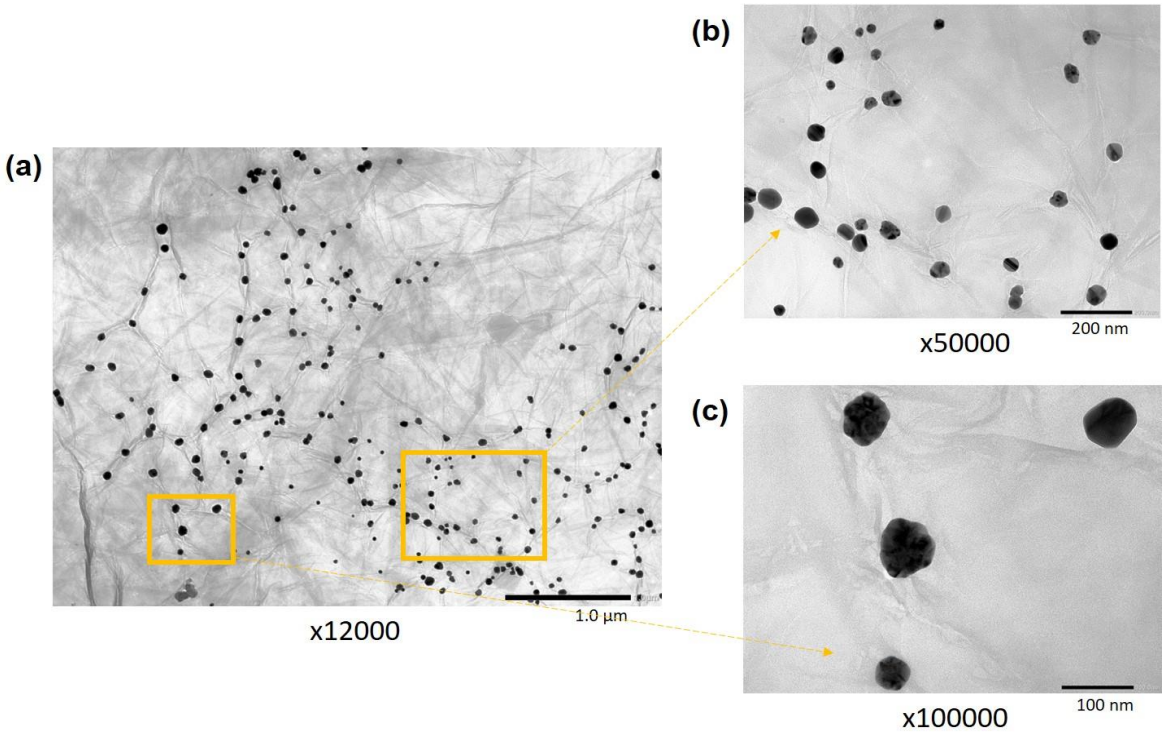


Figure 6-8 TEM images of Ag nanoparticles on the graphene oxide sheets at different magnifications and scale bars (a; x12k / 1µm, b; x50k / 200nm, c; x100k / 100nm).

6.3.3 Effect of irrigants on microbial killing

6.3.3.1 Main canal sampling point (MAIN)

NaOCl 2.5% presented the lowest values of detectable viable counts (log_{10}CFU) and was significantly more effective in microbial killing than the other irrigants, regardless of irrigant delivery method (P<0.05) (Fig. 6-9).

Ag-GO, NaOCl 1% and CHX 2% presented similar efficacy, which was significantly higher than sterile saline and EDTA 17% (P<0.05) (Fig. 6-9). The application of UAI or XPEF did not significantly affect the number of detectable viable counts (log_{10}CFU) compared to CI (P>0.05) (Fig. 6-9).

Negative control group specimens presented non-detectable viable counts (log_{10}CFU).

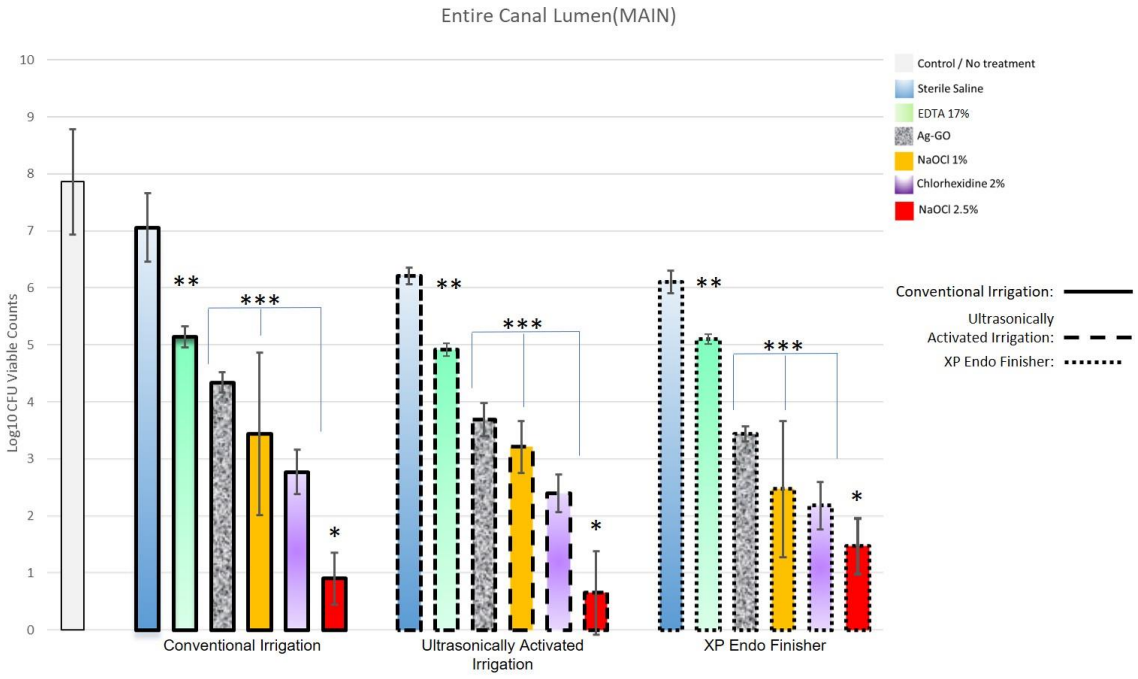


Figure 6-9 The effect of microbial killing of root canal irrigants and agitation methods in the main canal lumen (MAIN). The data are expressed as mean(SD) detectable anaerobic quantitative viable counts (log_{10}CFU).

* Values significantly less compared to rest experimental groups (*P*<0.05). ** Values significantly less compared to sterile saline groups (*P*<0.05).*** Values significantly less compared to EDTA 17% and sterile saline groups (*P*<0.05) and no statistically significant differences at their inter-group comparisons (*P*>0.05).

6.3.3.2 Middle lateral canal sampling point (LAT1)

Sterile saline, EDTA 17%, CHX 2% and NaOCl 1% presented limited microbial killing efficacy, which was not statistically different from the positive control group (P>0.05) (Fig. 6-10).

Ag-GO (UAI) and NaOCl 2.5% (CI), (UAI) enhanced microbial killing efficacy compared to the rest of the experimental groups (P<0.05) (Fig. 6-10).

The application of XPEF deteriorated the microbial killing efficacy of Ag-GO and NaOCl 2.5% and viable counts (log_{10}CFU) were not statistically different compared to sterile saline and positive control groups (P>0.05) (Fig. 6-10).

Negative control group specimens presented non-detectable viable counts (log_{10}CFU).

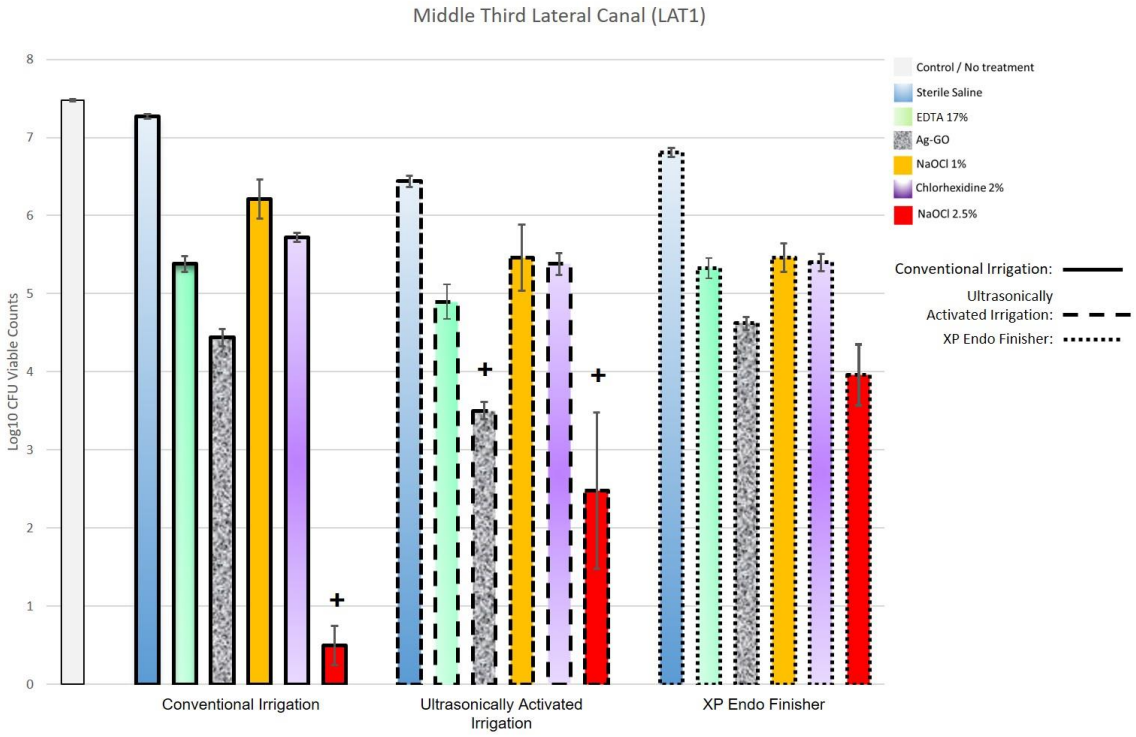


Figure 6-10 The effect of microbial killing of root canal irrigants and agitation methods in the middle third lateral canal (LAT1). The data are expressed as mean(SD) detectable anaerobic quantitative viable counts (log_{10}CFU).
[+]Values significantly less compared to rest experimental groups (*P*<0.05).

6.3.3.3 Apical lateral canal sampling point (LAT2)

NaOCl 2.5% presented non-detectable viable counts (log_{10}CFU), regardless of the applied irrigant delivery method. The application of UAI significantly improved the microbial killing efficacy of Ag-GO compared to CI and XPEF, as well as compared to NaOCl 1% (UAI), (XPEF) and CHX 2% (UAI), (XPEF) (P<0.05).

Limited microbial killing efficacy was observed with the application of CHX 2% (CI), (UAI), with no statistical difference compared to sterile saline and control groups (P>0.05) (Fig. 6-11). The application of XPEF significantly improved the performance of CHX 2% compared to CHX 2% (CI), (UAI) (P<0.05) (Fig. 6-9). CHX 2% (XPEF), NaOCl 1% (XPEF) and Ag-GO (XPEF) did not present significant differences in their viable counts (log_{10}CFU) (P>0.05) (Fig. 6-11).

All negative control group specimens presented non-detectable viable counts (log10CFU).

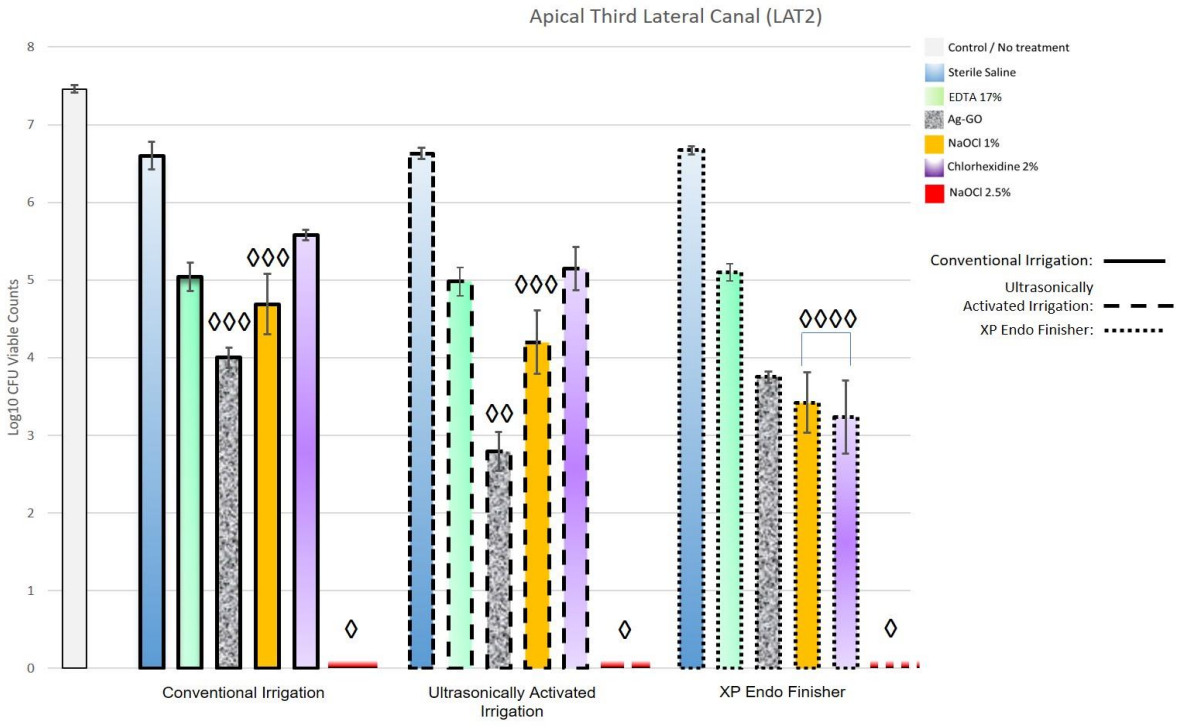


Figure 6-11 The effect of microbial killing of root canal irrigants and agitation methods in the apical third lateral canal (LAT2). The data are expressed as mean(SD) detectable anaerobic quantitative viable counts (log_{10}CFU).

◊ N/D: non-detectable values of anaerobic quantitative viable counts (log10CFU). ◊◊ Values significantly less compared to rest experimental groups (P<0.05). ◊◊◊ Values significantly less compared to EDTA 17 (CI), CHX 2% (CI), EDTA 17% (UAI), CHX 2% (UAI) (P<0.05). ◊◊◊◊ Values significantly less compared to NaOCl 1% (CI), CHX 2% (CI), NaOCl 1% (UAI) and CHX 2% (UAI) groups (P<0.05).

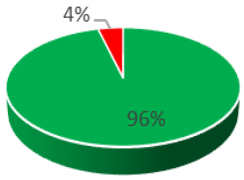
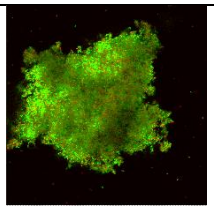
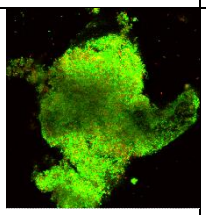
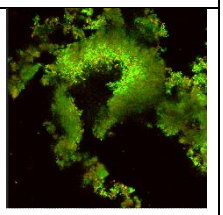
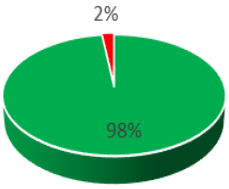
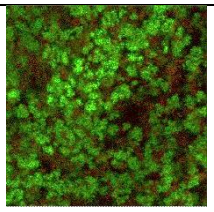
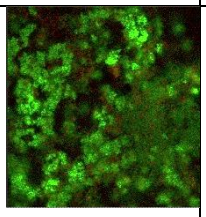
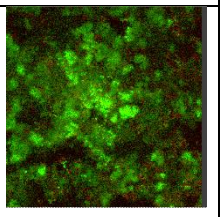
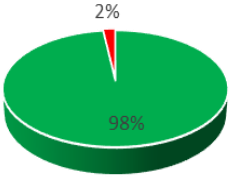
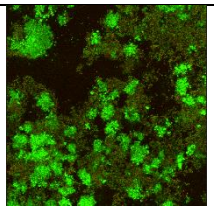
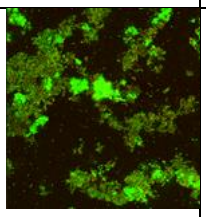
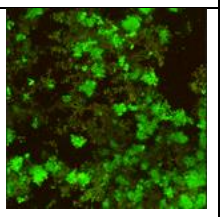
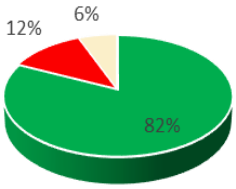
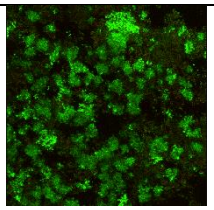
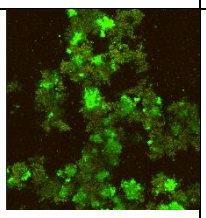
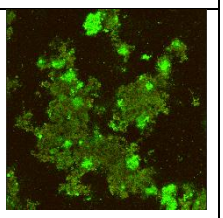
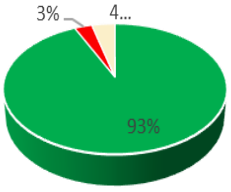
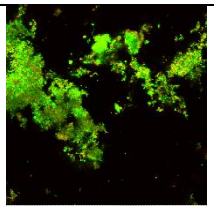
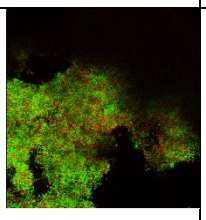
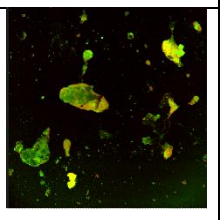
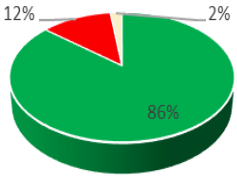
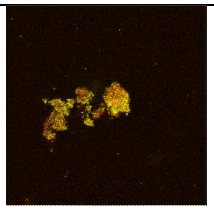
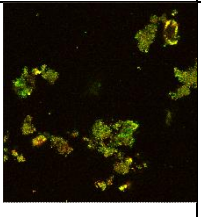
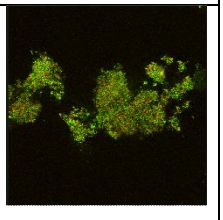
6.3.4 Effects of irrigants on biofilm disruption

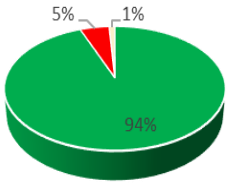
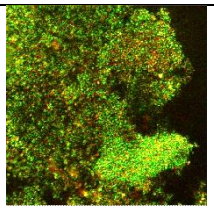
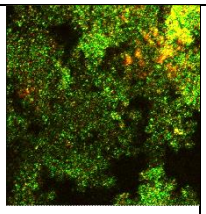
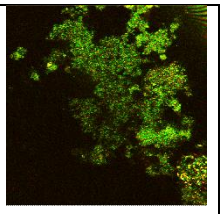
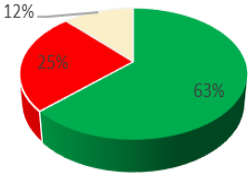
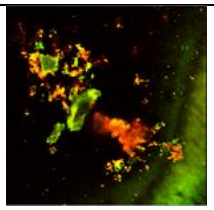
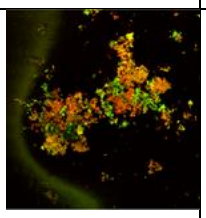
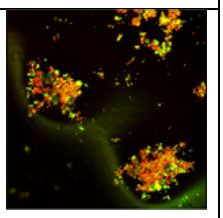
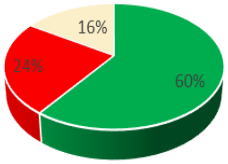
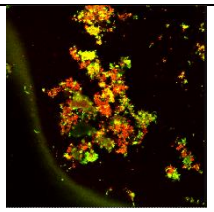
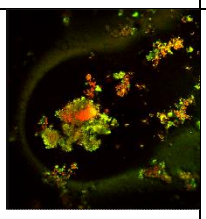
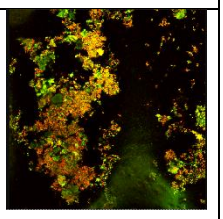
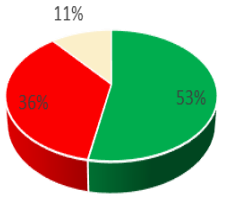
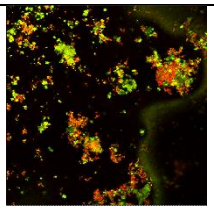
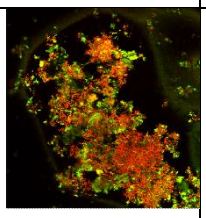
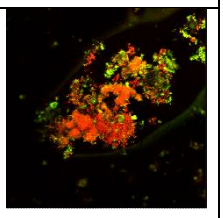
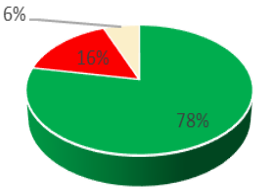
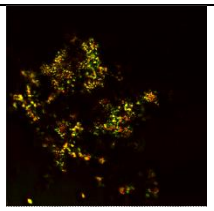
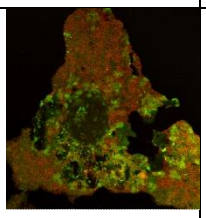
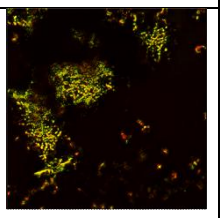
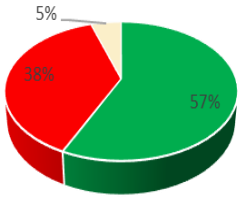
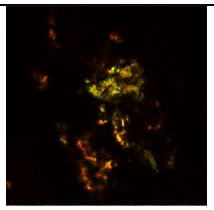
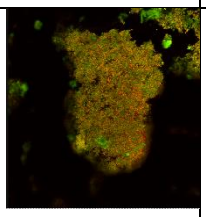
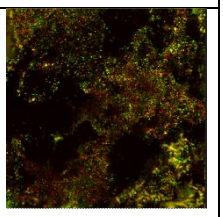
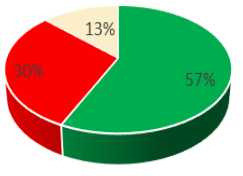
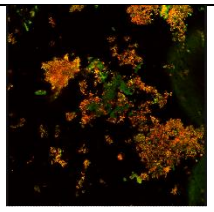
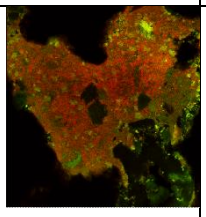
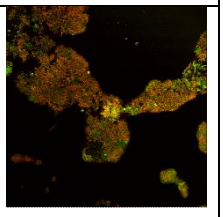
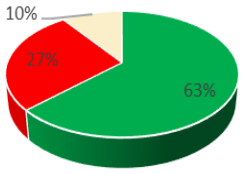
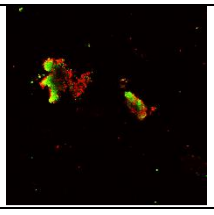
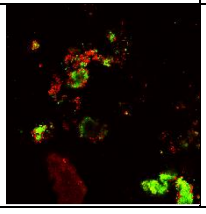
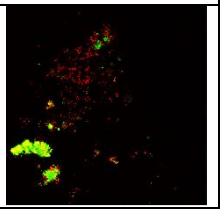
The effects of irrigants and their agitation techniques on biofilm disruption, in the main and lateral canals of the tooth specimens are presented in Table 6-2. All treatment groups presented significantly less total biovolume compared to the positive control group (no treatment) ($P< 0.05$). The maximum biofilm disruption was achieved by NaOCl 2.5% (CI), (UAI) and no viable biovolumes were present due to absence of green or red fluorescence. With the application of XPEF, a residual biovolume was present only in middle lateral canals with a mean percentage of 32% representing red (dead) biovolume.

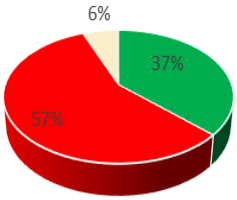
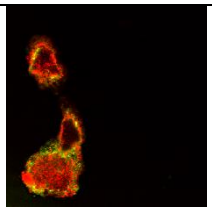
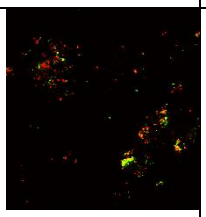
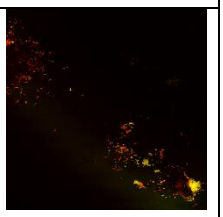
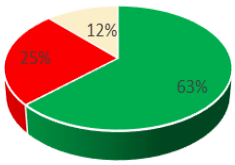
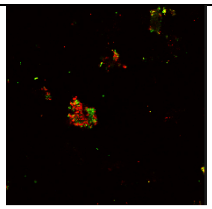
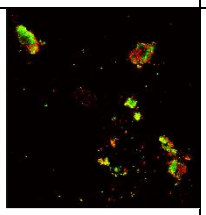
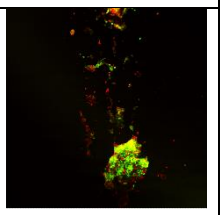
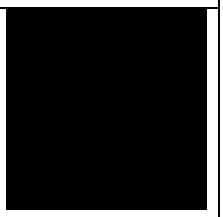
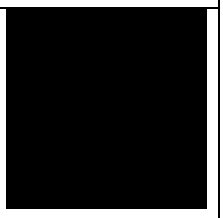
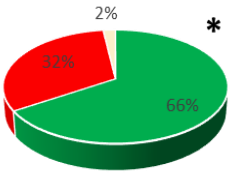
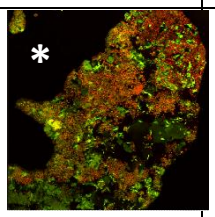
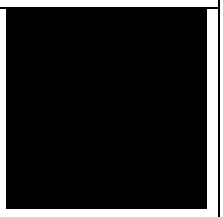
Ag-GO presented significant reduction of total biovolumes compared to the rest experimental groups, regardless of irrigation/agitation method ($P<0.05\%$). The application of UAI enhanced its biofilm disruption capacity with a mean percentage of 57% representing red (dead) biovolume, which was statistically significant compared to CI (27% red biovolume) and XPEF (25% red biovolume) ($P<0.05$).

The application of UAI significantly improved the disruption efficacy of EDTA 17% and NaOCl 1%, compared to CI and XPEF, respectively, as well as compared to CHX 2% (CI), (UAI), (XPEF) ($P<0.05$). However, EDTA 17% (UAI) did not present enhanced killing activity, with a mean percentage of 86% representing green (live) biovolume. With the use of NaOCl 1% (UAI), the enhancement of biofilm disruption efficacy was associated with an increase in the mean percentage of red (dead) biovolume (38%), which was statistically significant compared to CI (16% red biovolume).

Table 6-2 The effect of irrigation and agitation methods on biofilm disruption. The results represented the remaining mean (SE) values of total biovolume (um^3/um^2) and their respective mean percentages of dead (red), live (green) and unknown (orange) microbial populations. Representative confocal images of residual biofilms from the 3 examined areas are also presented.

Irrigant type / Irrigation Activation method		Total biovolume (um^3/um^2) Mean (SE)	Percentage mean of total biovolume Green: Live Red: Dead Orange: unknown	Location within root canal system		
				Main root canal (apical third)	Middle lateral canal	Apical lateral canal
Positive control	N/T	300369 (128447) [a]				
Sterile Saline	CI	180221 (104961)				
	UAI	212291 (32532)				
	XPEF	218844 (43284)				
EDTA 17%	CI	125175 (88862)				
	UAI	60582 (27376) [b]				

	XPEF	164373 (22490)				
CHX 2%	CI	104710 (13275)				
	UAI	128888 (44671)				
	XPEF	133646 (53546)				
NaOCl 1%	CI	107791 (73570)				
	UAI	45670 (29459) [c]				
	XPEF	180431 (131649)				
Ag-GO	CI	26182 (8374) [d]				

	UAI	17272 (7050) [d,e]				
	XPEF	22283 (7296) [d]				
NaOCl 2.5%	CI	N/D				
	UAI	N/D				
	XPEF	152583 (50861) *Detectable in middle root lateral canal				

N/T: No treatment

[a]: Mean (SE) total biovolume values significantly increased compared to all experimental groups (P<0.05).

[b]: Mean (SE) total biovolume values from the use of EDTA 17% (UAI) significantly less compared to EDTA 17% (CI), (XPEF) (P<0.05).

[c]: Mean (SE) total biovolume values from the use of NaOCl 1% (UAI) significantly less compared to NaOCl 1% (CI), (XPEF) (P<0.05).

[d]: Mean (SE) total biovolume values from the use of Ag-GO (CI), (UAI), (XPEF) significantly less compared to their respective in EDTA 17%, CHX 2% and NaOCl groups (P<0.05).

[e]: Mean (SE) total biovolume values from the use of Ag-GO (UAI) significantly less compared to Ag-GO (CI), (XPEF) (P<0.05).

6.4 Discussion

Various experimental models have been proposed to test the antimicrobial activity of root canal irrigants, in simulated clinically relevant dynamic conditions. These include 3-D printed transparent methacrylate (Mohmmed *et al.* 2016) or PEG-modified PDMS root models (Layton *et al.* 2015), transparent models from solidified polydimethylsiloxane (Macedo *et al.* 2014), intact root specimens (Azim *et al.* 2016, Van der Waal *et al*. 2015) or adjustable hemi-sected root halves (Bhuva *et al.* 2010, Lin *et al.* 2013, Niazi *et al.* 2014). A novel *ex vivo* tooth model was developed in order to improve stability and retention of hemi-sected root specimens and minimise the effect of thickness reduction, following the use of the microtome. The tooth model was reproducible and allowed for the simulation of an *ex vivo* closed apical system, to avoid fundamental methodological limitations from not simulating periradicular tissue pressure (Psimma *et al.* 2013). Thus, the compilation of a closed apical and lateral canal system was selected to avoid overestimation of irrigant flow or extrusion (Psimma *et al.* 2013), which could potentially lead to generation of false positive quantitative data. An *in vitro* nutrient-stressed, multi-species biofilm was successfully established on longitudinally hemisected root halves similarly to a previous study (Niazi *et al.* 2014).

In single-rooted teeth, lateral canals branch from the main, with diameters ranging from a minimum of 10 up to 400μm (Miyashita *et al.* 1997). Artificial lateral canals with varying diameters of more than 0.12mm have been drilled in decalcified natural teeth (Smith *et al.* 2000) or resin blocks (Dulac *et al.* 1999, Silver *et al.* 1999). In addition, artificial lateral canals with internal diameters varying from 0.25mm to 0.3mm were constructed in a transparent solidified polydimethylsiloxane model (Macedo *et al.* 2014) and 3-D printed transparent root blocks (Mohmmed *et al.* 2018), respectively.

To the authors' best knowledge, this is the first *ex vivo* tooth model, in which, the created artificial canals were characterised and quantified, ensuring their fabrication is predictable, repeatable and reliable. We also describe the first use of NCLP in the dimensional analysis (x, y, z) of artificially created features, within an *ex vivo* tooth model used to test endodontic irrigation efficacy. The use of 3-D step height to quantify z-dimensional data, according to ISO 5436-1, has been previously described as an appropriate method for quantifying step-heights and valleys,

which is what these artificial lateral canals can be classified as, within the context of surface metrology (Mylonas *et al.* 2018). Previous studies creating artificial lesions, whether lateral canals, internal resorption defects, or other intra- / extra-canal channels have only described the method of lesion creation, but not the lesion itself, assuming the dimensions of said features to be correct (van der Sluis & Wesslink 2007, Ulusoy *et al*. 2018). Accurate dimensional analysis of any intra-canal features, whose dimensions may affect the behaviour and flow of endodontic irrigants, must be analysed once they are created to ensure they kept consistent across all samples; any samples should be ideally rejected from the study if they do not meet dimensional requirements. Thus, the efficacy of irrigation was evaluated in standardized apical and middle root third artificial lateral canals.

Real-time recording of biofilm fluorescence (Mohmmed *et al.* 2016), analysis with confocal laser scanning microscopy (CLSM) (Niazi *et al.* 2014, Azim *et al.* 2016), scanning electron microscopy (SEM) (Bhuva *et al.* 2010, Lin *et al.* 2013), MTT assay (Van der Waal *et al.* 2015) and quantitative measurement of viable counts following paper point sampling (Niazi *et al.* 2014, Van der Waal *et al.* 2015) have been considered as acceptable methods to test microbial killing or biofilm disruption efficacy. Each methodology has inherent limitations and the proper selection should be based on the scopes of a study, to examine the antimicrobial capacity of irrigants in microscopic or macroscopic level.

Microbiological root canal sampling has been commonly employed to assess the effectiveness of endodontic treatment measures (Dalton *et al.* 1998). However, sampling procedures are limited in inaccessible regions of the root canal system, such as fins, accessory canals and isthmi (Bhuva *et al.* 2010). Two artificial lateral canals were prepared and standardised in apical and middle root thirds, in order to overcome this drawback (Niazi *et al.* 2014). Paper point sampling was adopted in order to examine the dynamic effects of irrigant disinfection in a simulated model within its macroscopic anatomical features. However, dentine is a ‘‘porous’’ biomaterial and dentinal tubules can be penetrated and colonised by bacteria up to 500μm in depth (Taschieri *et al.* 2014). Confocal laser scanning microscopy (CLSM) was also performed to quantify the biofilm disruption capacity of the irrigants on dentinal tubules surfaces, at three different sampling points. The 3-D visualization and quantification of the residual biovolume of live

or dead bacteria, *in situ*, were crucial factors to justify the distribution and viability, as well as the extension of the antibacterial effect of the irrigants.

All tested irrigants were subjected to challenging environmental conditions, associated with no instrumentation but direct exposure against the biofilm, application of limited irrigant volume, low flow rate, as well as short exposure time. This was decided with a view to assess the effect of irrigant delivery and activation methods and reduce the carry-over antimicrobial effect of concentrated solutions.

Sterile saline had no effect on microbial killing. Although a significant reduction in total biovolume was observed compared to positive control group under CLSM, no difference was found in live (green) biofilm viability. This finding suggests that microbial killing cannot be achieved with agitation methods only; thus, the use of a chemical active disinfectant is essential. As a chelating agent, EDTA 17% can bind calcium ions, which are essential for cell co-adherence and stabilization of extracellular polymeric substance of a biofilm (Chen *et al.* 2000, Chávez de Paz *et al.* 2010). Thus, the moderate biofilm disruption observed on application of UAI can be attributed to its biofilm-dispersing properties (de Almeyda *et al.* 2016). The role of EDTA in biofilm detachment and root canal cleaning has not been elucidated yet and requires further investigation.

The present study showed that application of Ag-GO (UAI) resulted in significantly improved microbial killing efficacy compared to CI and XPEF, in the artificially prepared lateral canals. The use of CLSM further disclosed a significant reduction in total microbial biovolume on dentine surfaces, compared to all experimental groups, except NaOCl 2.5%, and regardless of irrigant delivery/activation method. The use of Ag-GO (UAI) contributed to the reduction of total biovolume and furthermore significantly increased the percentage of dead (red signal) bacteria up to 57%. In the main canal lumen, the microbial killing efficacy of Ag-GO was similar to levels achieved with NaOCl 1% and CHX 2%. In the apical third lateral canal, the use of NaOCl 2.5% (CI, UAI, XPEF) resulted in non-detectable microbial counts. The high concentration and the increased apical shear wall stress may enhance the elimination of apical lateral canal biofilm (Layton *et al.* 2015). The antimicrobial efficacy of Ag-GO (UAI) in lateral canals was superior to that of NaOCl 1%, CHX 2%, EDTA 17% and sterile saline.

The use of Ag NPs has been reported to display minor biofilm disruption capacity compared to NaOCl, in root canal dentine sections examined by CLSM (Wu *et al.* 2014). Previous studies also indicate that the rate of bacterial killing by nanoparticles depend on the concentration and duration of interaction (Kishen *et al.* 2008, Shrestha *et al.* 2010, Zhuang *et al.* 2011), hence the limited effectiveness of sole irrigation with Ag NPs is attributed to the resistance of the biofilm matrix and the short time period of root canal irrigation (Rodriguez *et al.* 2018). Other studies report that sole Ag NPs may not be stable in suspension to exert their antimicrobial activity due to agglomeration, resulting from the resistance of some bacteria, which can produce the adhesive flagellum protein flagellin, which triggers aggregation of the Ag NPs (Panáček *et al.* 2018).

The multi-potent mechanism of antimicrobial action of GO has gained attention, especially as the bi-dimensional GO sheets can act as cutters of cell membranes. In particular, the sharp edges of GO can mechanically disrupt bacterial membranes leading to leakage of the intracellular cytoplasm, increase of reactive oxygen species and cell death (Akhavan *&* Ghaderi 2010, Liu *et al.* 2011, Combarros *et al.* 2016). GO sheets can also make a flexible web or blanket around microorganisms, envelop and wrap around the cell surfaces without penetration. This isolates them from the external environment and nutrients, leading to inactivation of proliferation and reduction in glucose consumption (Akhavan *et al.* 2011). In addition, the antimicrobial properties of GO are also associated to its low pH values especially if GO is not subjected to purification procedures that allows soluble acidic impurities to remain within the milieu (Barbolina *et al.* 2016).

Hence the incorporation of Ag NPs within GO sheets to form an aqueous Ag-GO suspension was tested for the first time for root canal disinfection using a nutrient-stressed, multispecies biofilm developed in an infected tooth model, *ex vivo*. The mechanism of Ag-GO formation has been attributed to the presence of oxygenic functional groups, such as hydroxyl, epoxide and carboxyl, on the GO, which provide a large number of binding sites for Ag ions (Zhu *et al.* 2013). Following the *in situ* reduction by $NaBH_4$ in aqueous solution, the positively charged Ag^+ ions can be easily captured by negatively charged GO surface through physicosorption, electrostatic binding and charge-transfer interactions (Hui *et al.* 2014). The hybrid material of Ag NPs within a matrix of a layered material like GO can induce binding capability, which is usually lacking in Ag NPs alone, and enhance its synergistic antimicrobial activity compared to sole GO

or Ag NPs (Liu *et al.* 2018). Thus, a stable Ag-GO nanocomposite was formed to overcome aggregation and stability.

Several studies have shown that compared to conventional irrigation, the application of UAI or XPEF enhances the efficacy of NaOCl in reducing intra-canal bacteria and removing biofilms from the main canal lumen, the apical third root surface and shallow or deep layers of root dentine (Azim *et al.* 2016, Alves *et al.* 2016). In this study, the application of UAI or XPEF did not improve microbial killing compared to conventional syringe irrigation, when Ag-GO, CHX 2% and NaOCl 1% or 2.5% were used. The differences can be attributed to different methodologies, irrigant concentrations and volume as well as total time/flow rate.

The antimicrobial efficacy of Ag-GO (UAI) was superior to that of NaOCl 1%, CHX 2%, EDTA 17% and sterile saline, in the apical third lateral canal. The use of XPEF did not improve the microbial killing efficacy of Ag-GO compared to UAI, but improved the performance of NaOCl 1% and CHX 2%. Our results are in agreement with a previous study, in which XPEF was found to be more effective compared to UAI and CI in removing biofilms from artificial apical lateral grooves (Bao *et al.* 2017). However, the exact mode of action of XPEF on irrigant delivery across a shaped root canal system needs further investigation.

The biofilm in middle root third lateral canal was least affected by the irrigants and the agitation methods used had limited effects, when NaOCl 1% (CI, UAI, XPEF), CHX 2% (CI, UAI, XPEF) and NaOCl 2.5% (XPEF) were used. This finding may be related to several parameters arising due to the dynamic conditions, which exist coronally to the insertion depth of the irrigation needle. This results in reduction of shear wall stress, leading to limited contact of irrigant with biofilm layer; low velocity of irrigant exchange with an absence of developing jet are also associated with the limited antimicrobial performance in middle lateral canal (Macedo *et al.* 2014, Robinson *et al.* 2017). Finally, the increased length of a lateral canal in the middle root third combined with limited irrigant penetration may further predispose for poor disinfection outcomes.

A significant reduction in the microbial counts of the middle root third lateral canal was observed with NaOCl 2.5% (CI), (UAI). The enhanced dissolution capacity against the organic matrix of attached biofilms may allow for easier disruption and killing of microbes at the interface and the cross-sectional area of the lateral canal (Macedo *et al.* 2014). In addition, the intermittent

ultrasonic activation procedures appear to increase the reactivity rate of NaOCl 2.5%, due to its increased chlorine availability after consecutive replenishment (Macedo *et al.* 2014b). Faster chlorine consumption can be potentially relevant to higher biofilm disruption capacity (del Carpio-Perochena *et al.* 2015).

The efficacy of Ag-GO (UAI) in middle lateral canal was similar to NaOCl 2.5% (UAI) and superior compared to the rest of the experimental groups. These findings can be attributed to a potentially existing, dual and synergistic mechanism of action of Ag-GO. The increased formation of reactive oxygen species has the ability to react with various biomacromolecules, causing irreversible oxidative damage, loss of cellular DNA replication ability and cell death (Feng *et al.* 2000). The enhancement of Ag-GO microbial killing and biofilm disruption efficacy with the use of UAI may correlate to a substantial increase in the velocity of the Ag-GO nanosheets against the exposed biofilm layers and cause an increase in temperature, which may enhance the kinetics of the Ag-GO nanosheets on a molecular level. The significant reduction in biofilm biovolume may also result from the wrapping capacity of Ag-GO sheets around ruptured biofilms and their detachment from dentinal tubules (Afkhami *et al.* 2011), with the aid of the developing jet when UAI is applied.

The application of Ag-GO for root canal irrigation as an alternative to NaOCl is promising, however studies are required to further elucidate the mechanism of action against endodontic biofilms. GO can be used as a platform for the safe delivery of inter-appointment medicaments that promote periapical healing as well as biomineralisation. Additional studies will be necessary to test Ag-GO formulation as inter-appointment medication as well as other applications in restorative dental materials and in bone graft substitutes. Potential drawbacks that may arise could relate to their possible deposition within dentine tubules and the risk of obliteration; thus, obstruction of adhesive techniques. Coronal or root discolouration should not be underestimated if GO suspension is not completely removed from the pulp chamber. Although Ag-GO presents low cytotoxicity and high biocompatibility (Cai *et al.* 2012, Zhang *et al.* 2015), the interaction with periapical tissues requires further study.

6.5 Conclusion

Within the limitations of this study, the microbial killing and biofilm disruption capacity of Ag-GO was successfully achieved using a novel *ex vivo* infected tooth model. The efficacy of irrigation protocols was affected by the sampling point areas of the infected root canal model. NaOCl 2.5% presented superior antimicrobial efficacy at all sampling sites and it was found that the middle root third lateral canal was the least affected sampling point area. Ultrasonic activation selectively enhanced the microbial killing and biofilm disruption of Ag-GO in lateral canals.

Chapter 7 General discussion, conclusions & suggestions for future work

7.1 General discussion

The main objective of root canal treatment is to preserve a tooth functional in the oral cavity and eliminate intra-canal bacteria, as a result of pulpal, periapical disease or dental trauma. In the United States of America, nearly 22.3 million people are treated annually via root canal treatments and more than 41.000 root canals are performed each day to retain oral function and avoid extraction of a diseased tooth (https://www.aae.org/specialty/about-aae/news-room/endodontic-facts/ AAE). Although the success rate of root canal treatment is high, the complex anatomy of the root canal system in conjunction with presence of resilient pathogenic bacteria forming resistant biofilms, the limitations of current chemo-mechanical instrumentation, irrigation and obturation methods often limit the microbiological and healing outcome of the procedure. The loss of teeth can lead to loss of oral function and appearance, which impacts ultimately on function, output and quality of life. This presents the dental practitioner with the challenges of disinfecting root canals effectively and safely, via the delivery of strong disinfectants such as NaOCl and auxiliary substances such as EDTA. Irrigation of the infected root canal system with 1-5% NaOCl is considered as a gold standard for decades now and is fairly successful in maintaining previously infected teeth healthy in the oral cavity (Basrani & Haapasalo 2012). The use of 17% EDTA, as an adjunct for removal of the inorganic phase of the produced smear layer during instrumentation, is also adopted as a good practice standard, despite the questionable effect of smear layer in the endodontic treatment outcomes (Haapasalo *et al.* 2012).

Meanwhile, the current research focus in endodontics relates to the improvement of the biocompatibility of materials and health and safety issues in practice, for the delivery of high-quality dental services. However, root canal irrigation with NaOCl and its interaction with the components of an infected root canal system has not received much attention. The review of the relevant background literature identified that there is a significant lack in the knowledge of

secondary chemical reactions of NaOCl, except from the basic chemical reaction, which only justify its antimicrobial and dissolution capacity. The concept of our research was based on the existing evidence of several water research and analytical chemistry studies, that claim the formation of toxic volatile components (VOCs) and disinfection by-products (DBPs) from the interaction of several chlorinated disinfectants with sources of natural organic matter (NOM) (Krasner 2009).

The compilation of this dual-phase study came as a result of the collaboration of a group of dental clinicians who specialise in the provision of endodontic (root canal) treatments and accredited scientists in the fields of microbiology, dental materials and analytical chemistry. During all experimental procedures, dentine powder samples and root specimens were subjected to sterilisation procedures via autoclaving. Pilot studies were conducted and confirmed that sterilisation procedures did not have any impact on the organic content of dentine, including collagen depletion (Ioannidis *et al.* 2016). Therefore, the mechanical and chemical treatment of dentine involved in endodontic procedures, as the main presenting hard tissue, was similar to clinically relevant conditions. The sterilisation of root specimens was also important for their use as appropriate biological substrates for biofilm growth. The experimental procedures were performed under aseptic conditions, to prevent cross-contamination of the specimens and undesirable chemical interactions. As a result, the risk of other chemical substances and external pathogens involvement was limited and did not hinder the experimental outcomes of sampling and chemical analysis procedures.

Chapter 3 has been a continuation of the conducted proof of concept studies described in Chapter 2. Hence, we report the validation, suitability and application of selected ion flow tube-mass spectrometry (SIFT-MS) for the detection of volatile compounds (VOCs) and disinfection by-products (DBPs). The chemical interaction of NaOCl with biomaterials of dental origin, including dentine powder, bacteria, serum albumin and their combination resulted in the formation of toxic DBPs and VOCs under aerobic and anaerobic conditions.

Taking on board the acquired knowledge and the potentials from sample chemical analysis with SIFT-MS, we intended to extend our search into tooth specimens *ex vivo*, in an attempt to simulate conditions of good clinical practice. In Chapters 4 & 5, a novel *ex vivo* tooth model was

designed to examine the formation of VOCs and DBPs during chemomechanical preparation of artificially infected root canals with rotary files, 2.5% NaOCl and 17% EDTA. Two areas of potential emission were examined, including a water-closed periradicular space and the collected effluent from the use of a portable medical suction. Therefore, the risk of apical extrusion and the probability of environmental effects were discussed. This is the first study to examine the formation of VOCs and DBPs in dentistry as a result of a standard and indispensable procedure such as root canal preparation and irrigation. The application of a powerful and easy-to-use analytical method such as SIFT-MS has proved an excellent analytical tool to study the chemical interactions.

The results of the studies proved that the mechanical preparation and irrigation of artificially infected root canals with rotary NiTi files, 2.5% NaOCl and 17% EDTA resulted in the formation of VOCs and DBPs in a water-closed periradicular space as well in aspirated effluent aliquots. An unexpected and novel finding was the formation of high concentrations of formaldehyde, a known carcinogen, which was further investigated and verified that originated from the chemical interaction between NaOCl and EDTA. The adsorptive capacity of Ag-AC selectively reduced the concentration of chloroform, but had no effect against ammonia, methanol and formaldehyde. From a clinical perspective, a first step to prevent the formation of such hazardous substances is to abort the intermittent use of NaOCl and EDTA, should this concept be currently taught or clinically recommended. The principles of root canal irrigation and any relevant chemical interactions should be taught to both undergraduate and postgraduate dental students. Furthermore, the concept of chelation and removal of smear layer should be reconsidered, with the introduction of less hazardous agents.

Part of the main objectives of this PhD was to propose a new method for the disinfection of root canals. In Chapter 6, we presented the first study in the field of dental materials utilising a nano silver-graphene oxide system (Ag-GO) to enhance a disinfection regimen against a nutrient-stressed multi-microbial endodontic biofilm with the aid of different agitation methods. We further presented the fabrication of a novel human tooth model, which simulates the dynamic conditions during root canal irrigation. Additionally, we reported, for the first time, the use of non-contacting laser profilometry (NCLP) to characterise artificially prepared lateral canals in human root specimens.

This study outlined the importance of implementing a novel disinfectant (Ag-GO) to overcome the limitations, health and occupational hazards from the use of NaOCl, which is still considered a gold standard. The irrigation of Ag-GO was performed with the aid of syringe delivery and two mechanical agitation methods, including ultrasonic activation and the use of a new file, known as XP Endo finisher. The antimicrobial effect of Ag-GO was studied with the aid of microbial sampling with paper points and measurement of colony forming units and biofilm disruption was studied with the aid of confocal laser scanning microscopy. The combination of the two methodologies provide a detailed insight into microbial reduction within the main root canal as well as lateral canals, which are difficult-to-reach areas in terms of irrigation replenishment.

Historically, the use of silver nanoparticles (Ag NPs) in Endodontics did not result in favourable disinfection outcomes due to the limitation of previous studies in characterisation and preparation techniques, as well as limited understanding of appropriate delivery methods in root canals. Graphene oxide (GO) has gained significant attention in literature due to its unique properties to bind molecules and act as a platform for their delivery in several domains in biomedical sciences. A significant challenge which has faced dental and materials researchers is to study new methods in the delivery of antimicrobial agents within root canal dentine surface and new approaches to disrupt or eliminate endodontic biofilms. Hence, the implementation of Ag-GO has proved effective because it combines the known antimicrobial and physicochemical properties of both materials.

7.2 Conclusions

The following conclusions can be drawn:

1. The use of different NaOCl concentrations (1%, 2% and 5.25%) had significant effects against the subsurface organic and inorganic degradation within mineralised dentine. Increased dentine powder weight and reduced volume of irrigant had an inhibitory effect on collagen depletion when NaOCl 1% and 2% concentrations were applied. NaOCl 5.25% exhibited the strongest effects on dentine collagen depletion regardless of dentine powder mass and irrigant

volume. In addition, NaOCl 5.25% presented moderate effects against the inorganic content of dentine.

2. The chemical interaction of human dentine with NaOCl 2.5% and 5% v/v resulted in the formation of toxic DBPs at different observation periods varying from 30sec to 90min. DBPs may also pre-exist as precursors within blank NaOCl solutions. NaOCl concentration and time did not have any effect on the degree of DBPs formation.
3. SIFT-MS is shown to be an effective technique for the real-time analysis of volatile compounds released by the reaction of NaOCl and representative components of an infected root canal system. The chemical interaction of NaOCl 2.5% with dentine powder, bacteria, bovine serum albumin and their combination resulted in the formation of toxic DBPs and VOCs under both aerobic and anaerobic conditions.
4. The mechanical preparation and irrigation of artificially infected root canals with rotary NiTi files, 2.5% NaOCl and 17% EDTA resulted in the formation of toxic VOCs and DBPs in a water-closed periradicular space. The chemical interaction of NaOCl and EDTA resulted in the generation of high concentrations of formaldehyde. The formation of chloroform and formaldehyde indicate that risk assessment of the potential hazards to health should be carried out.
5. The mechanical preparation and irrigation of artificially infected root canals with rotary NiTi files, 2.5% NaOCl and 17% EDTA resulted in the formation of VOCs and DBPs in aspirated effluent aliquots. Dental units are not supplied with POU treatment or purification systems, as far as chemically derived effluents are concerned. The potential environmental hazards from dental liquid waste have not been determined yet and their management remains unregulated. The chemical interaction of NaOCl and EDTA resulted in high concentrations of formaldehyde release. The adsorptive capacity of Ag-AC selectively reduced the concentration of chloroform, but had no effect against ammonia, methanol and formaldehyde.
6. The microbial killing and biofilm disruption capacity of Ag-GO was successfully achieved using a novel *ex vivo* infected tooth model. The efficacy of irrigation protocols was affected by the sampling point areas of the infected root canal model. NaOCl 2.5% presented superior antimicrobial efficacy at all sampling sites and it was found that the middle root third lateral

canal was the least affected sampling point area. Ultrasonic activation selectively enhanced the microbial killing and biofilm disruption of Ag-GO in lateral canals.

7.3 Suggestions for future work

The risks and drawbacks from the daily use of large quantities of NaOCl in endodontic procedures require further analysis and critical appraisal with additional environmental and human exposure criteria. Further clinical studies are required for the examination of the cumulative effects on dental staff and the degree of patient exposure through inhalation of the volatile phase of DBPs during endodontic treatment procedures, under conditions of good practice.

The results of the present PhD may open a new prospective for the assessment of professional and environmental hazards and risks in dental practice, following the potential exposure of patients, dental clinicians and staff on a daily basis. Environmental implications should not be under-estimated also. The future objective will be to adapt these data and perform additional human studies to assess risks of extrusion of VOCs and DBPs in clinical dental settings as well as the degree of environmental effects of the effluent chlorinated aliquots, which do not receive any point-of-use purification and are discharged in waste-water pipelines, enhancing further the phenomenon of bio-accumulation of VOCs and DBPs.

The synthesis and application of Ag-GO provided a further understanding of its antimicrobial properties in a crucial dental application, such as the irrigation of infected root canals. Ag-GO may be a promising alternative to NaOCl, however studies are required to further elucidate the mechanism of action against endodontic biofilms. This may be of great interest to dentists and scientists in the fields of dental antimicrobials, dental nanotechnology and endodontic microbiology, as it will allow for development of additional formulations and relevant *in vitro* models, to study the beneficial antimicrobial properties of Ag-GO in other dental domains.

GO can be used as a platform for the safe delivery of inter-appointment medicaments that promote periapical healing, periodontal regeneration as well as biomineralisation. Additional studies will be necessary to test Ag-GO formulation as inter-appointment medication as well as other applications in restorative dental materials and in bone graft substitutes. Potential

drawbacks that may arise could relate to their possible deposition within dentine tubules and the risk of obliteration; thus, obstruction of adhesive techniques. Coronal or root discolouration should not be underestimated if GO suspension is not completely removed from the pulp chamber.

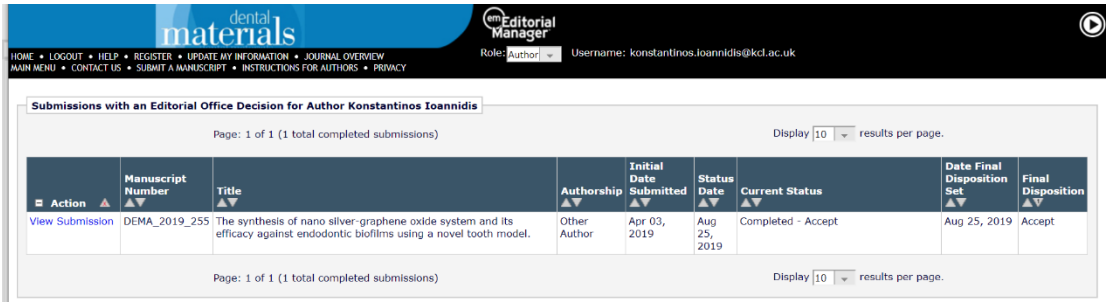
Appendices

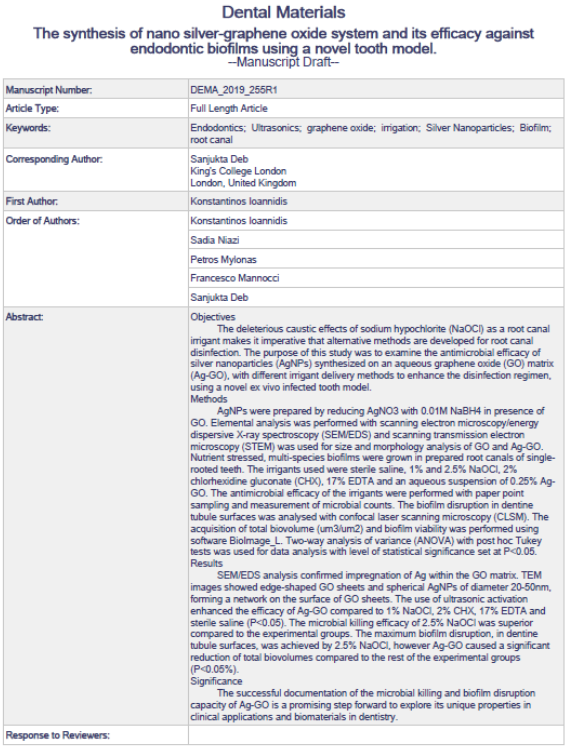
Appendix I: Publications

List of publications in international peer-reviewed journal

Ioannidis, K., Niazi, S., Deb, S., Mannocci, F., Smith, D. and Turner, C. (2018). Quantification by SIFT-MS of volatile compounds produced by the action of sodium hypochlorite on a model system of infected root canal content. PLOS ONE, 13 (9), e0198649.

Ioannidis, K., Niazi, S., Mylonas, P., Mannocci, F., Deb, S. (2019). The synthesis of nano silver-graphene oxide system and its efficacy against biofilms using a novel tooth model. Dental Materials, *ACCEPTED FOR PUBLICATION*





Dental Materials

The synthesis of nano silver-graphene oxide system and its efficacy against endodontic biofilms using a novel tooth model.

--Manuscript Draft--

Manuscript Number:	DEMA_2019_255R1
Article Type:	Full Length Article
Keywords:	Endodontics; Ultrasonics; graphene oxide; irrigation; Silver Nanoparticles; Biofilm; root canal
Corresponding Author:	Sanjukta Deb King's College London London, United Kingdom
First Author:	Konstantinos Ioannidis
Order of Authors:	Konstantinos Ioannidis
	Sadia Niazi
	Petros Mylonas
	Francesco Mannocci
	Sanjukta Deb
Abstract:	Objectives The deleterious caustic effects of sodium hypochlorite (NaOCl) as a root canal irrigant makes it imperative that alternative methods are developed for root canal disinfection. The purpose of this study was to examine the antimicrobial efficacy of silver nanoparticles (AgNPs) synthesized on an aqueous graphene oxide (GO) matrix (Ag-GO), with different irrigant delivery methods to enhance the disinfection regimen, using a novel ex vivo infected tooth model. Methods AgNPs were prepared by reducing AgNO3 with 0.01M NaBH4 in presence of GO. Elemental analysis was performed with scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDS) and scanning transmission electron microscopy (STEM) was used for size and morphology analysis of GO and Ag-GO. Nutrient stressed, multi-species biofilms were grown in prepared root canals of single-rooted teeth. The irrigants used were sterile saline, 1% and 2.5% NaOCl, 2% chlorhexidine gluconate (CHX), 17% EDTA and an aqueous suspension of 0.25% Ag-GO. The antimicrobial efficacy of the irrigants were performed with paper point sampling and measurement of microbial counts. The biofilm disruption in dentine tubule surfaces was analysed with confocal laser scanning microscopy (CLSM). The acquisition of total biovolume (um3/um2) and biofilm viability was performed using software BioImage_L. Two-way analysis of variance (ANOVA) with post hoc Tukey tests was used for data analysis with level of statistical significance set at P<0.05. Results SEM/EDS analysis confirmed impregnation of Ag within the GO matrix. TEM images showed edge-shaped GO sheets and spherical AgNPs of diameter 20-50nm, forming a network on the surface of GO sheets. The use of ultrasonic activation enhanced the efficacy of Ag-GO compared to 1% NaOCl, 2% CHX, 17% EDTA and sterile saline (P<0.05). The microbial killing efficacy of 2.5% NaOCl was superior compared to the experimental groups. The maximum biofilm disruption, in dentine tubule surfaces, was achieved by 2.5% NaOCl, however Ag-GO caused a significant reduction of total biovolumes compared to the rest of the experimental groups (P<0.05%). Significance The successful documentation of the microbial killing and biofilm disruption capacity of Ag-GO is a promising step forward to explore its unique properties in clinical applications and biomaterials in dentistry.
Response to Reviewers:	

Full Length Article | DEMA_2019_388

Ex vivo detection and quantification of apically extruded volatile compounds and disinfection by-products by SIFT-MS, during chemomechanical preparation of infected root canals

Francesco Mannocci, Claire Batty, Claire Turner, David Smith, Konstantinos Ioannidis, Sanjukta Deb

Submitted 13 May 2019

With Editor 13 May 2019

View details

List of abstracts submitted to national and international conferences

- K. Ioannidis, R. Busquets, S. Deb & F. Mannocci (Poster Presentation-R61), **'Detection of toxic chlorinated by-products from sodium hypochlorite interaction with dentine. Preliminary report.'** European Society of Endodontology, 17th Biennial ESE Congress - Barcelona, Spain, 16–19 September 2015. [Abstract] published in International Endodontic Journal **49**, page 59, 2016.

- Ioannidis K, Niazi S, Deb S, Mannocci F, Smith D, Turner C (Oral Presentation 0473), '**Formation of Volatile Disinfection By-products During Endodontic Irrigation With Sodium Hypochlorite'** Submitted for ***BSODR Prize Competition Voco Prize for Dental Biomaterials Research (sponsored by Voco),*** International Association of Dental Research, 96th General Session of the IADR in conjunction with the IADR Pan European Regional Congress, London, UK, 25-28 July 2018.

- Konstantinos Ioannidis, Sadia Niazi, Sanjukta Deb, Francesco Mannocci, David Smith, Claire Turner (Poster Presentation WP 189), **'Use of SIFT-MS in the analysis of volatile compounds arising from the addition of sodium hypochlorite as an irrigant in infected root canal treatment'** Division of Mass Spectrometry (DSM) of the Italian Chemical Society, 22nd International Mass Spectrometry Conference, Florence, Italy, 26-31 August 2018.

- Konstantinos Ioannidis, Sadia Niazi, Petros Mylonas, Francesco Mannocci, Sanjukta Deb (Oral Presentation), **'The synthesis of a nano silver-graphene oxide system and efficacy against endodontic biofilms using a novel tooth model'** Royal Society of Chemistry, Biomaterials Chemistry Annual Conference, Liverpool, UK, 9-11 January 2019.

- Konstantinos Ioannidis, Claire Batty, David Smith, Sanjukta Deb, Francesco Mannocci, Claire Turner (Oral Presentation), **'The use of SIFT-MS in understanding the production of potentially toxic VOCs during routine root canal procedures'** The Second International Conference on Soft Chemical Ionisation Mass Spectrometry and its Applications to Trace Gas Analysis, ICSCIMS 2019, J. Heyrovsky Institute of Physical Chemistry of the CAS, Prague, Czech Republic, 10–13 June 2019.

- Ioannidis K, Batty C, Turner C, Deb S, Mannocci F (Poster Presentation), **'*Ex vivo* detection and quantification of volatile compounds and disinfection by-products by SIFT-MS, during chemomechanical preparation of infected root canals'**

European Society of Endodontology, 19th Biennial ESE Congress - Vienna, Austria, 12-14 September 2019.

Appendix II: Research Ethics Committee approval letter

Wales REC 4
G1/G2 Croesnewydd Hall
Croesnewydd Road
Wrexham Technology Park
Wrexham LL13 7YP

Telephone : 01978 726377

E-mail : tracy.biggs@wales.nhs.uk

Website : www.nres.nhs.uk

06 June 2014

Professor Francesco Mannocci
Professor in Endodontology, Hons Consultant
King's College London,Dental Institute
Floor 25 Guy's Tower, Restorative Division
SE1 9RT

Dear Professor Mannocci

Study title:	**The effect of irrigation with sodium hypochlorite on the formation of chlorinated disinfection by-products (DBPs) in the root canal system and periradicular space during instrumentation.**
REC reference:	**14/WA/1004**
Protocol number:	**N/A**
IRAS project ID:	**152590**

Thank you for your letter of 04 June 2014 responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The following revised documentation has been reviewed and approved by the sub-committee:

Letters of invitation to participant	2	04 June 2014
Participant information sheet (PIS)	2	04 June 2014
Participant consent form	2	04 June 2014

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Mrs Tracy Biggs, Tracy.Biggs@Wales.nhs.uk.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

- **Participant Information Sheet – What will happen to the samples I give?**

  Please specify extracted teeth. Currently "biological samples" is stated then the mention of extracted teeth as an example. As the study is specifically about extracted teeth this should be explicit to avoid a patient/participant being confused.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents
The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Evidence of Sponsor Insurance or Indemnity (non NHS Sponsors only) [SPONSOR INSURANCE]	V.1	29 July 2013
IRAS Checklist XML [Checklist_21052014]		21 May 2014
Other [2ND SUPERVISOR BRIEF CV]	V.1	
REC Application Form [REC_Form_21052014]		21 May 2014
Research protocol or project proposal [RESEARCH PROTOCOL]	V.1	31 March 2014

Summary CV for Chief Investigator (CI) [BRIEF CV FRANCESCO MANNOCCI]	V.1	21 May 2014
Summary CV for student [CV RESEARCH STUDENT]	V.1	21 May 2014
Summary CV for supervisor (student research) [BRIEF CV FRANCESCO MANNOCCI]	V.1	21 May 2014
Letters of invitation to participant	2	04 June 2014
Participant information sheet (PIS)	2	04 June 2014
Participant consent form	2	04 June 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/WA/1004	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

T.A. Biggs.

Professor Alex Carson
Chair
E-mail: tracy.biggs@wales.nhs.uk

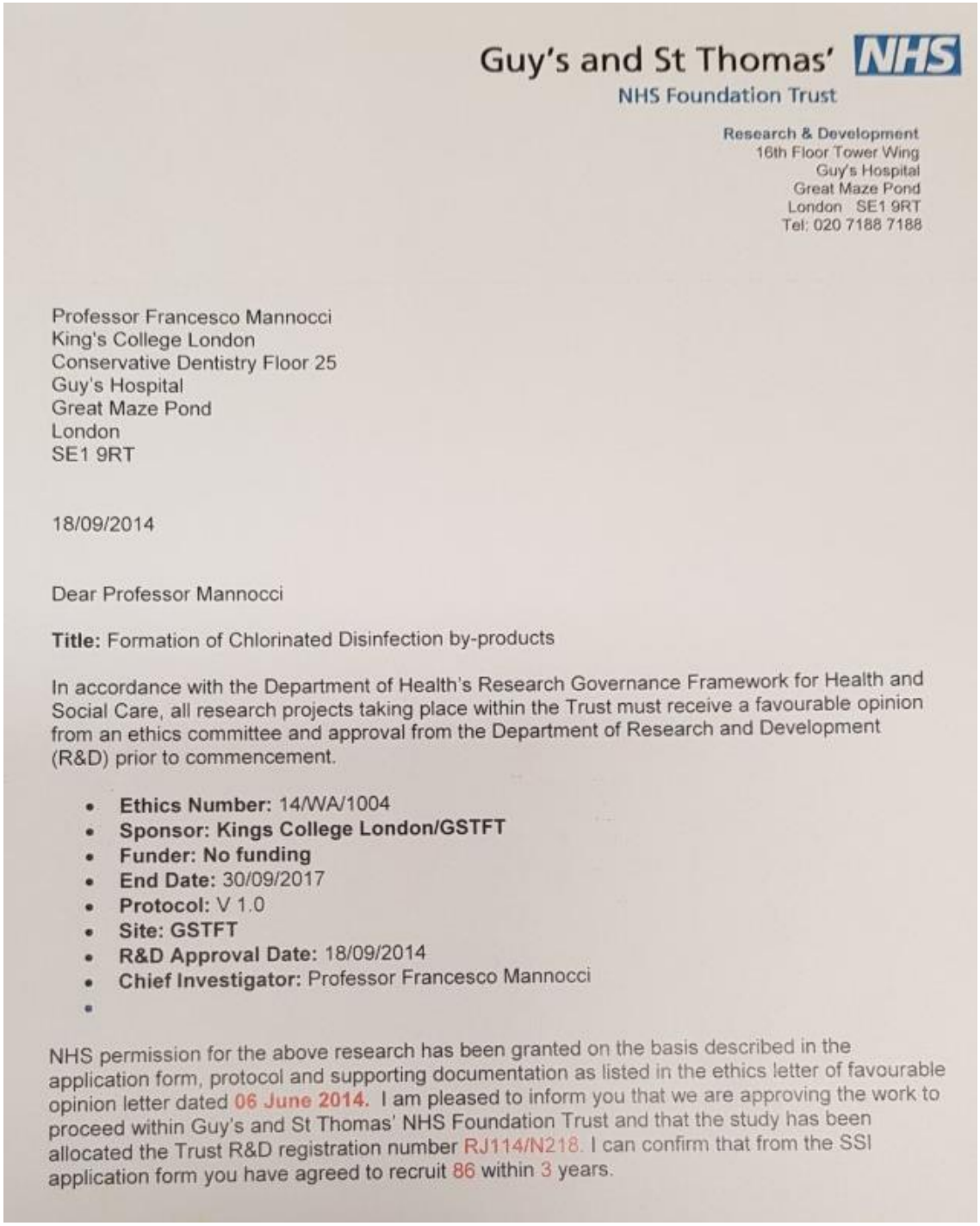
Enclosures: *"After ethical review – guidance for researchers"*

Copy to: *Sponsor - Dr Keith Brennan*

R& D Dept - Ms Karen Ignatian, Guy's and St Thomas' Hospital Trust

Student – Dr Kostas Ioannidis

Appendix III: Research and Development approval letter



Guy's and St Thomas' NHS
NHS Foundation Trust

Research & Development
16th Floor Tower Wing
Guy's Hospital
Great Maze Pond
London SE1 9RT
Tel: 020 7188 7188

Professor Francesco Mannocci
King's College London
Conservative Dentistry Floor 25
Guy's Hospital
Great Maze Pond
London
SE1 9RT

18/09/2014

Dear Professor Mannocci

Title: Formation of Chlorinated Disinfection by-products

In accordance with the Department of Health's Research Governance Framework for Health and Social Care, all research projects taking place within the Trust must receive a favourable opinion from an ethics committee and approval from the Department of Research and Development (R&D) prior to commencement.

- **Ethics Number:** 14/WA/1004
- **Sponsor: Kings College London/GSTFT**
- **Funder: No funding**
- **End Date:** 30/09/2017
- **Protocol:** V 1.0
- **Site: GSTFT**
- **R&D Approval Date:** 18/09/2014
- **Chief Investigator:** Professor Francesco Mannocci
-

NHS permission for the above research has been granted on the basis described in the application form, protocol and supporting documentation as listed in the ethics letter of favourable opinion letter dated **06 June 2014.** I am pleased to inform you that we are approving the work to proceed within Guy's and St Thomas' NHS Foundation Trust and that the study has been allocated the Trust R&D registration number RJ114/N218. I can confirm that from the SSI application form you have agreed to recruit 86 within 3 years.

Conditions of Approval:

- The principal investigator must ensure that the recruitment figures are reported.
- The principal investigator must notify R&D of the actual end date of the project.
- R&D must be notified of any changes to the protocol prior to implementation.
- The project must follow the agreed protocol and be conducted in accordance with all Trust Policies and Procedures especially those relating to research and data management.
- Members of the research team must have appropriate substantive or honorary contracts with the Trust prior to the study commencing. Any additional researchers who join the study at a later stage must also hold a suitable contract.

Data Protection:
Please ensure that you are aware of your responsibilities in relation to The Data Protection Act 1998, NHS Confidentiality Code of Practice, NHS Caldicott Report and Caldicott Guardians, the Human Tissue Act 2004, Good Clinical Practice, the NHS Research Governance Framework for Health and Social Care, Second Edition April 2005 and any further legislation released during the time of this study.

The Principal Investigator is responsible for ensuring that Data Protection procedures are observed throughout the course of the project.

If the project is a clinical trial under the European Union Clinical Trials Directive the following must also be complied with:

1. The EU Directive on Clinical Trials (Directive 2001/20/EC) and UK's implementation of the Directive: The Medicines for Human Use (Clinical Trials) Regulations 2004;
2. The EU Directive on Principles and Guidelines for Good Clinical Practice (EU Commission Directive 2005/28/EC); and UK's implementation of the Directive: The Medicines for Human Use (Clinical Trials) Amendment Regulations 2006;
3. If a clinical trials team has to keep a subject in a department" out of hours" for whatever reason, the Senior Nurse for the Hospital should be informed of their presence – as should the Resuscitation Team.
4. For CtiMP studies hosted by GSTFT, the sponsor is responsible for reporting updates and providing updated documents related SMPC at this site

Amendments:
Please ensure that you submit a copy of any amendments made to this study to the R&D Department.

ISRCTN registration:
If appropriate it is recommended that you register with the Current Controlled Trials website http://isrctn.org/. Find out more about registering for an International Standard Randomised Controlled Trial Number (ISRCTN) as part of the Portfolio application process. Non-commercial studies with an interventional component that are eligible for NIHR CRN support can register for an ISRCTN for free via the Portfolio Database.

Annual Progress Report:
It is obligatory that an annual report is submitted by the Chief Investigator to the research ethics committee, and we ask that a copy is sent to the R&D Department. The yearly period commences from the date of receiving a favourable opinion from the ethics committee.

Please submit a copy of the progress report on the anniversary of the Ethics favourable opinion **6th June.**

In line with the Research Governance Framework, your project may be randomly selected for monitoring for compliance against the standards set out in the Framework. For information, the Trust's process for the monitoring of projects and the associated guidance is available from the Trust's intranet or on request from the R&D Department. You will be notified by the R&D Department if and when your project has been selected as part of the monitoring process. No action is needed until that time.

Should you require any further information please do not hesitate to contact us.

Yours sincerely

Rachel Fay
R&D Governance Specialist

Appendix IV: Participant's information sheet

Kostas Ioannidis
DDS, MSc,
Specialist in Endodontics
MPhil / PhD Student
Tel: 07414979270
Email:konstantinos.ioannidis@kcl.ac.uk

Dept of Biomaterials
Floor 17; Tower Wing
Guy's Hospital
London
SE1 9RT

Guy's and St Thomas' NHS
NHS Foundation Trust

Francesco Mannocci
Professor, Hons. Consultant
Tel: 02071881573
Email: francesco.mannocci@kcl.ac.uk

Restorative Division
Department of Endodontology
Floor 25; Tower Wing
Guy's Hospital
London
SE1 9RT

PARTICIPANT INFORMATION SHEET

Title of study:

The effect of irrigation with sodium hypochlorite on the formation of chlorinated disinfection by-products (DBPs) in the root canal system and periradicular space during instrumentation.

REC reference number: 14 / WA / 1004
IRAS PROJECT ID: 152590

Dear Sir / Madam,

When you attend for your scheduled dental operation, you may be approached to take part in a laboratory study. We would cordially ask you to give 3-5 minutes and read the following information with regard to your potential participation. Thank you in advance.

What is the purpose of the study?

Our study is an approved MPhil / PhD in Pre-Clinical Dentistry research project.
The purpose of our laboratory study is to look into what chemical substances are produced when we apply a commonly used disinfectant (sodium hypochlorite) for dentine surface and root canal disinfection. Root canal disinfection is an essential dental procedure which is carried out inside the tooth in case it requires endodontic treatment (root canal treatment).

Why have I been chosen?

Because you are having a tooth removed and we would like to carry out some laboratory tests on it before disposal. There will be no genetic testing.

What will happen to the samples I give?

As you have been referred for hospital dental care, we would like your permission to collect biological samples, such as *EXTRACTED TEETH*.

If you decide to take part you will be asked to sign a *CONSENT FORM*.

Please note the treatment you have been planned for and receive WILL NOT BE AFFECTED by this study. In addition, there are no additional risks in the quality of your clinical care which correlate to this study.

We will not use any personal or confidential data. Kostas Ioannidis is responsible to keep the tooth sample anonymized; thus, it will non-identifiable and no one will be able to retrieve back to your personal details and confidential data.

After the completion of your dental treatment, your participation for the purposes of this study finishes.

The biological samples (extracted tooth/teeth) will be collected by a qualified member of staff, transferred to the researcher (Kostas Ioannidis) who is the person in charge to store it anonymized in safe environment (Laboratory of Biomaterials, Floor 17, Guy's Tower).

It is hoped this study will provide valuable insight into chemical reactions during root canal treatment procedures, the identification of any formed undesirable by-products and possible measures to prevent their emergence.

Samples will be destroyed following the end of this project and disposed in line with Human Tissue Act.

You can stop taking part in the research at any time. This will not affect the care you will receive.

Who has reviewed this study?

All research in the NHS is reviewed by a Research Ethics Committee to protect your interest. This study has been reviewed by Wales REC 4.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. Please contact: Principle

Investigator Professor Francesco Mannocci (email: francesco.mannocci@kcl.ac.uk, telephone: 02071881573).

If you have a complaint, you should talk to your research doctor who will do their best to answer your questions. If you remain unhappy, you may be able to make a formal complaint through the NHS complaints procedure. Details can be obtained through the Guy's and St Thomas' Patient Advisory Liaison Service (PALS) on 0207 1888188, address: PALS, KIC, Ground floor, north wing, St Thomas' Hospital, Westminster Bridge Road, London, SE1 7EH.

This trial is co-sponsored by King's College London and Guy's and St Thomas' NHS Foundation Trust. The sponsors will at all times maintain adequate insurance in relation to the study independently. Kings College London, through its own professional indemnity (Clinical Trials) and no fault compensation and the Trust having a duty of care to patients via NHS indemnity cover, in respect of any claims arising as a result of clinical negligence by its employees, brought by or on behalf of a study patient.

Thank you for taking the time to read this invitation to take part in this study.

If you have any questions about this project please contact Dr. Kostas Ioannidis

Yours sincerely,

Dr Christopher Sproat

BDS(Lond), FDSRCS(Eng), MD, BS(Hons)(Lond)

Consultant in Oral Surgery, Department of Oral Surgery,

Floor 23, Tower Wing, Guy's Hospital, London, SE1 9RT

Appendix V: Patient's consent form

Kostas Ioannidis
DDS, MSc,
Specialist in Endodontics
MPhil / PhD Student
Tel: 07414979270
Email:konstantinos.ioannidis@kcl.ac.uk
Dept of Biomaterials
Floor 17; Tower Wing
Guy's Hospital
London
SE1 9RT

Francesco Mannocci
Professor, Hons. Consultant
Tel: 02071881573
Email: francesco.mannocci@kcl.ac.uk
Restorative Division
Department of Endodontology
Floor 25; Tower Wing
Guy's Hospital
London
SE1 9RT

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON
University of London

REC reference number: 14 / WA / 1004
IRAS PROJECT ID: 152590

CONSENT FORM

Title of Project:

The effect of irrigation with sodium hypochlorite on the formation of chlorinated disinfection by-products (DBPs) in the root canal system and periradicular space during instrumentation.

Name of Researchers:

Chief Investigator: Professor Francesco Mannocci

Postgraduate Research Student: Dr Kostas Ioannidis

Please initial all boxes

1. I confirm that I have read and understand the information sheet dated **[4/6/2014]** (version **[V.2]**) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

1. I understand that relevant sections of my medical notes and data collected during the study, will not be looked at from the NHS Trust, where it is relevant to my taking part in this research. ☐

2. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent.	Date	Signature

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